WP T1. Deliverable 3.3

Report on active/viable biological actors

(WPT1_Activity 3_Deliverable 3.3)

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Table of contents

1. Introduction

Biowastes derived mainly from organic sources such as sewage sludge, food/plant waste, animal manures and other animal by-products are certain to contain a high concentration of a variety of micro-organisms, some of which may include pathogens, particularly fecal material. Pathogens are essentially micro-organisms capable of causing disease, and those found in such materials may cause subclinical infection, as well as serious infections, which may even be deadly. These infections may occur through direct exposure in handling and processing, or via indirect routes such as agricultural produce and water runoff. While some pathogens are species-specific, others are zoonotic, thus, posing risks to public health.

Several factors influence the survival of pathogens during the treatment processes that biowastes can be subjected to. For example, in biogas plants, parameters influencing the pathogen survival in anaerobic digestion process include temperature and time, which are the most critical, as well as other factors such as pH, volatile fatty acids, whether batch or continuous digestion is used, the inital number of pathogens contained within the biowaste, bacterial species and the nutrients available (Sahlstrom, 2003). Pathogenic bacteria of significant importance in organic wastes include *Salmonella spp., E.coli*, *Listeria spp.*, *Campylobacter spp.* and *Yersinia spp.,* among others. Additionally, such materials can be contaminated with both species-specific and zoonotic viruses, e.g. the hepatitis E virus, and parasites, e.g. Cryptosporidium.

The current EU Fertilising Products Regulation (FPR) (EU) 2019/1009, as set out by European Parliament, outlines the standards for pathogens in fertilizers, including organic, organo-mineral and inorganic fertilizer products and soil improvers. These fertilizer products should be tested for both *Salmonella spp.* and *Escherichia coli* or *Enterococcaceae*, where *Salmonella spp.* should be absent per 25g/25ml of fertilizer tested and *Escherichia coli* or *Enterococcaceae* should not exceed 1,000 CFU (colony forming units) per 1g/1ml of fertilizer.

The primary objective of this deliverable is to investigate the presence of pathogenic bacteria in recycling derived fertilizers (RDFs). It is intrinsic that the RDFs are devoid of potential pathogens that can give rise to health and safety concerns. Additionally, the presence of weeds in RDFs is investigated and total viable counts (TVCs) are carried out on the RDFs for an indication of the number of viable micro-organisms present within the RDFs.

1.1. Description of RDFs being tested

Fifteen different RDFs from different sources, using different processing techniques, were produced and provided by ReNu2Farm project partners. It is essential that these fertilizers conform to standards and do not pose health threats to humans or animals through contamination of foodstuffs or water bodies with pathogenic material.

Table 1. List of tested recycling-derived fertilizers (RDFs), and brief description of the production process that they were subjected to.

Product	Process description
Ash	Thermal incineration of sewage sludge by fluidized bed technology
Ash	Incineration of poultry litter
Bed ash	Gasification of sewage sludge and green cuttings by fluidized bed technology
Struvite	Produced from dewatering digested sludge from communal WWTP by crystallization with Mg by using the NuReSys reactor
Struvite	P precipitation with $FeCl_3$ by NuReSys technology on waste stream from potato processing company
Compost (L30)	Solid Fraction of pig slurry (30%) and poultry manure (70%) mix
Compost (L70)	Solid Fraction of pig slurry (70%) and poultry manure (30%) mix
Compost (L100)	Solid Fraction of pig slurry after anaerobic digestion and composting
Ammonium sulphate	H_2SO_4 chemical scrubbing of air from pig stables
Ammonium nitrate	Stripping and HNO_3 scrubbing of liquid fraction of digestate
Pig Urine	Stabling system of separating pig urine from pig manure
Ammonia water	LF of digestate (from food waste processing) is evaporated and the water is condensed in the form of so called ammonia water
Liquid concentrate	LF of digestate (from food waste processing) is evaporated and a concentrate of N and K is obtained
Blend 1	Mixture of ammonium nitrate and liquid concentrate
Blend 2	Mixture of liquid concentrate and ammonia water

2. Methods and materials

2.1. Enumeration of total bacterial and fungal cells

For a quantitative estimation of the number of mesophillic microorganisms in RDFs derived from sewage sludge, animal wastes and green cuttings, total viable counts (TVCs) were performed. The total viable count is concerned only with viable (or live), culturable bacterial or fungal cells that are capable of producing colonies when supplied with the nutrients required for growth. The colonies that grow are known as colony forming units (CFUs), with each single colony equalling one CFU. Ultimately, growth medium is inoculated with a series of tenfold serially diluted samples to achieve a number of colonies between 30-300, so they can be accurately counted with the naked eye. The number of colonies counted can then be multiplied by the dilution factors to calculate the number of CFUs per g/ml of sample. All work is carried out aseptically, and where possible, under a laminar flow cabinet, to avoid contamination of media or materials.

Total viable bacterial and fungal counts were carried out using Plate Count Agar and Malt Extract Agar, manufactured by LabM, which is selelctive for the enumeration of bacteria and fungi respectively. Media for enumeration of bacterial cells was supplemented with cycloheximide, a fungicide produced by the bacterium Streptomyces griseus, for the inhibiton of fungi, while media for enumeration of fungi was supplemented with kanamycin sulphate, an aminoglycoside bacteriocidal antibiotic isolated from Streptomyces griseus, to inhibit bacterial growth.

Typicla formula of Plate Count Agar:

Formula	g/L
Tryptone	5.0
Yeast extract	2.5
Glucose	1.0
Agar No. 1	15.0

pH 7.0 +/- 0.2

Typical formula of Malt Extract Agar:

Formula	g/L
Malt extract	30.0
Mycological peptone	5.0
Agar No. 2	15.0

pH 5.4 +/- 0.2

2.1.1. Bacteria

Media and sample preparation: For total viable bacterial counts, Plate Count Agar was prepared as per manufacturers specifications and autoclaved at 121°C, 15 psi. Once the molten agar had cooled to ~50°C, a pre-prepared 10mg/ml cycloheximide stock solution was added at a concentration of 10µg/ml. For total viable fungal counts, Malt Extract Agar was prepared as per manufacturers specifications and autoclaved at 121°C, 15psi. Once the molten agar had cooled to ~55°C, a 50mg/ml kanamycin stock solution was added at a concentration of 50µg/ml of agar. All agar media was kept in a water bath at 50°C so that it would remain molten. Quarter strength Ringer's solution was prepared as per manufacturers specifications, added to a series of 5 test tubes in 9mL aliquots and autoclaved. 1g of fertilizer was weighed and added to the first tube of 9ml Ringer's solution and vortexed to create a 10^{-1} dilution. Serial dilutions were prepared as far as 10^{-5}.

Figure 1. Prepared media solidifying (left) and RDF sample dilutions (right).

Inoculation, incubation and enumeration: The media was inoculated using the pour plate technique under a laminar air-flow cabinet for sterility. As a preliminary test to determine the serial dilution which yielded a countable number of CFUs in the range of 30 to 300, 1ml of each dilution was added to a series of empty petri dishes before pouring in the molten agar and quickly swirling the dishes to mix. The plates were left to solidify before placing in an incubator at 37°C for 48 hours. Once the incubation period was complete, the number of CFUs for each dilution were counted and the most appropriate dilution was chosen for further testing. The experiment was repeated with the chosen dilution in triplicate to determine the average number of CFUs and subsequently, calculate the average number of viable bacterial cells inhabiting one gram of fertilizer.

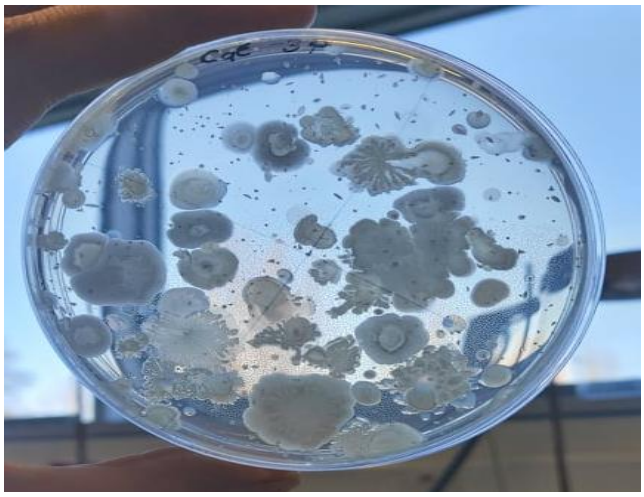

Figure 2. Example of total viable bacteria count (liquid concentrate on plate count agar).

2.1.2. Fungi

Enumeration of fungal cells in fertilizers was performed in the same manner as for bacteria, this time using Malt Extract Agar for enumeration of fungi, supplemented with kanamycin sulphate for bacterial inhibition. For total fungal counts, plates were incubated at 30°C for 7 days.

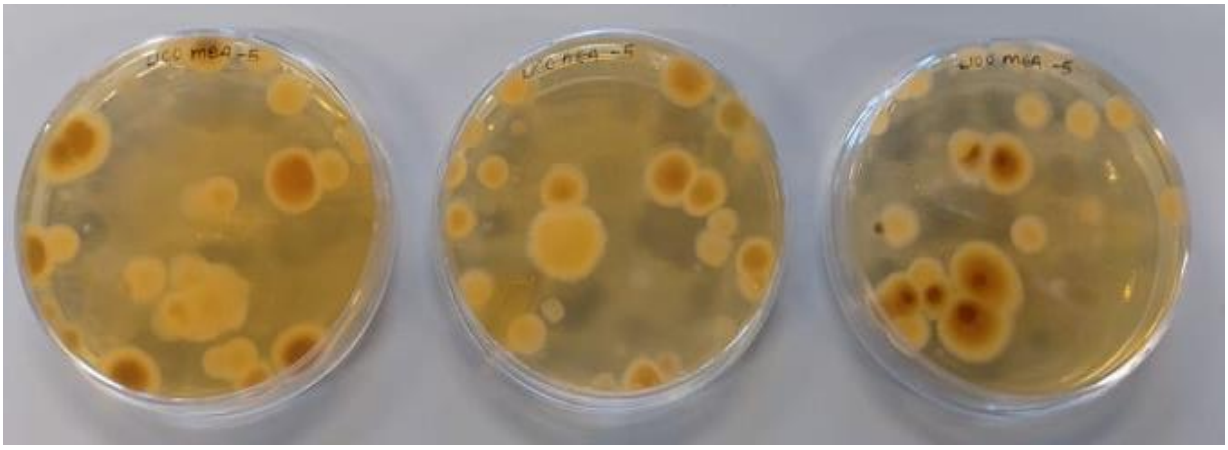

Figure 3. Example of total viable fungi counts (L100 compost on malt extract agar).

2.2. Test for presence of coliforms

Coliform bacteria are bacteria found in the digestive tracts and wastes of animals, and also in plant and soil material. Though coliforms are unlikely to cause illness, they are often used as indicator organisms for sanitary quality of foods and water, as their presence is indicative of fecal contamination, meaning enteric pathogens such as *Salmonella* may be present. The purpose of carrying out tests for coliforms here, was to establish whether, and which RDFs require further testing for specific pathogens. Tests for total coliforms and *Escherichia coli* were performed using the Colilert* test kit manufactured by IDEXX. The Colilert* kit detects both coliforms and *E.coli* simultaneously and is based on two nutrient-indicators, ortho-Nitrophenyl-β-galactoside (ONPG) and 4-methylumbelliferyl-beta-D-glucuronide (MUG), providing the major sources of carbon. Coliforms can be detected as they metabolise ONPG using the enzyme β-galactosidase, which causes the medium to change from colourless to yellow. The presence of *E.coli* is detected by its ability to metabolise MUG using β-glucuronidase which causes fluorescence. Other organisms are suppressed as most do not contain these enzymes. Those that do have the ability to grow are suppressed by the test kit's specifically formulated matrix. These test kits are usually used for the detection of coliforms/*E.coli* in water samples. Here, it was used to detect the presence of these organisms in a number of RDFs, by adjusting the method to suit this purpose. All work is carried out aseptically, and where possible, under a laminar flow cabinet, to avoid contamination of media or materials.

Samples were prepared by adding 1g/1ml of RDF in 99mL of autoclaved sterile water in Duran bottles and shaking vigorously to create 1 in 100 dilutions. This was repeated for all RDF samples. RDFs which caused the solution to

become opaque or added colour were diluted further by 1 in 10 to avoid inaccurate interpretation of results. The contents of a single Colilert* nutrient-indicator pack were added to each RDF dilution along with 1-2 drops of IDEXX antifoam solution and shaken. This was repeated using sterile water alone, triplesuper phosphate and calcium ammonium nitrate, serving as controls. The sample mixtures were poured into IDEXX Quanti-Trays* 2000 and sealed with an IDEXX Quanti-Tray* sealer before incubating at 35°C for 18 hrs. Following incubation the Quanti-Trays were removed from the incubators and observed colour change or fluoresnsce. The most probable number of total coliforms in each sample was calculated by counting the number of wells in the Quanti-Trays* which had changed from colourless to yellow. The same approach was taken for counting the most probable number of *E.coli*, but in a dark room under a 365nm UV lamp to observe fluorescent wells. The Colilert-18 test has a detection limit of 1 organism/100 mL for both coliforms and *E.coli*.

2.3. Tests for presence of specific pathogenic organisms

Investigations for the presence of pathogens, including *Salmonella, E.coli, Campylobacter and Listeria* were outsourced to another laboratory, Agrihealth Laboratory Services, Co. Monaghan, Ireland, due to the absence of a class 2 laboratory for handling these pathogens at ITC, and the impossibility to carry out these tests in a class 2 laboratory elsewhere due to the COVID-19 pandemic. Agrihealth Laboratory Services are accredited by the Irish National Accreditation Board (INAB) with ISO/IEC 17025 for biological and veterinary testing. The methods used for the detection of each pathogen and its corresponding ISO standard.

- Detection and isolation of *Salmonella spp.* per 25g of sample through selective enrichment technique. Based on ISO 6579- 1:2017 Microbiology of the Food Chain - Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella Part 1: Detection of *Salmonella Spp.*
- Detection of *Listeria spp.* per 25g of sample by the VIDAS UP (LPT) method using ELFA (Enzyme Linked Fluorescent Assay). Based on EN ISO 16140 validation study of the VIDAS UP Listeria method for the detection of *Listeria.*
- Enumeration of β-glucuronidase-positive *Escherichia coli* per g/ml sample by colony count technique. Based on ISO 16649-2- 2001 Horizontal Method for the enumeration of β-glucoronidase-positive *Escherichia coli* part 2: Colony count technique at 44 degrees using 5-bromo-4-chloro-3-indolyl β -D-glucuronide.

- Enumeration of *Campylobacter spp.* per g/ml sample by colony count technique. Based on ISO 10272-1:2017 - Horizontal Method for detection and enumeration of *Campylobacter spp.* - Part 2: Colony Count technique.

2.4. Test for the presence of weeds in RDFs

While the presence of potential weeds in RDFs is highly unlikely, except possibly in case of compost products, an experiment was carried out to confirm this. No official method is available to guide testing for the presence of viable weed seeds in RDFs. Therefore, a simple pot experiment was set up in which commercial compost was placed in pots as a growing medium for potential weeds and fertilized with RDFs to see if any weeds grow. The compost was autoclaved at 121°C, 15 psi to ensure the removal of any weed seeds and 100g of compost was placed in a series of pots with 1g/1ml of RDF. This was done in triplicate for each RDF. The pots were distributed randomly in a plant growth room kept at 21°C and were irrigated daily (where possible) for six weeks.

Figure 4. Pot trial setup for testing for the presence of weeds in RDFs.

3. Results and discussion

3.1. TVCs

Numbers of total viable bacterial cells were high in some RDF products, while others were completely devoid of bacteria, or had negligible numbers. In the case of fungi, all RDFs had no, or negligible numbers of fungi, with the exception of L100 compost.

Table 2. Average number and standard deviation of bacterial and fungal CFUs (n=3) for each fertilizer.

Fertilizer	Number of bacterial CFUs/g or ml	Standard Deviation	Number of fungal CFUs/g or ml	Standard deviation
Control (sterile water)	-	-	-	-
Triple super phosphate (TSP)	0.3×10^1	0.5	-	-
Calcium ammonium nitrate (CAN)	0.3×10^1	0.5	0.3×10^1	0.5
Struvite (sewage sludge processing)	1.4×10^2	4.3	7.6×10^2	6.5
Struvite (potato processing)	1.9×10^3	10.6	1.0×10^1	0.8
Ash (poultry litter)	-	-	-	-
Ash (sewage sludge)	-	-	-	-
Bed Ash (sewage sludge and green cuttings)	3.8×10^2	14.4	0.3×10^1	0.5
Ammonium nitrate	0.6×10^1	0.9	-	-
Ammonium sulphate	4.3×10^1	1.7	7.0×10^1	0.8
Ammonia water	-	-	-	-
Pig urine	2.1×10^3	92.9	1.0×10^1	0.8
Liquid concentrate	3.1×10^5	9.9	4.0×10^1	0.8
Blend 1	9.3×10^5	2.1	1.3×10^2	4.1
Blend 2	6.8×10^5	4.8	8.0×10^1	1.7
Compost (L30 = 30% pig slurry)	3.6×10^4	3.7	-	-
Compost (L70 = 70% pig slurry)	7.2×10^5	30.9	3.0×10^1	0.5
Compost (L100 = 100% pig slurry)	4.7×10^4	6.6	4.9×10^4	4.5

3.2. Coliforms

The results of tests for coliforms using IDEXX Colilert* kits were difficult to interpret. The reason being that some samples turned yellow, but fainter than for a positive result. These samples included blend 1, liquid concentrate, pig urine, struvite from sewage sludge, ammonium water and ammonium sulfate. Pig urine was the only sample to fluoresce under UV-light, which indicates the presence of *E.coli*, but again, this was not particularly strong.

Usually, this would be interpreted as a negative result. Furthermore, the entire sample turned a faint yellow (i.e. in all wells), which is unlikely to happen for such a small volume of sample and given the treatment processes they were subjected to. For this reason, a results table was not included. However, for absolute clarity, it was decided that the RDFs should be tested further to determine the presence of specific pathogens. Arrangements were made to carry out this work in University Limerick (UL), due to the lack of a Class 2 laboratory at Institute of Technology Carlow (ITC) for culturing such organisms. However, the COVID-19 pandemic meant this could not take place. Therefore, a company in Ireland named Agrihealth Limited agreed to carry out this work, the details and results of which are described in part 2.3 and 3.3, respectively. The decisions on which RDFs should be sent for further testing was based on two factors:

1. Those with a high number of bacterial CFUs per gram or milliliter of fertilizer, including liquid concentrate, blend 1, blend 2, L30, L70 and L100.
2. Those which displayed ambiguous results using the Colilert* kit (i.e. slightly yellow), as previously stated above.

Figure 5. RDF samples in Quanti-Trays after the 18-hour incubation period. From left to right, the samples are blend 1, sterile water, liquid concentrate, struvite from potato processing and pig urine. The faint yellow colour change is visible in blend 1, liquid concentrate and pig urine.

3.3. Pathogenic organisms

Results of detection and isolation methods revealed that no Salmonella or Listeria were present in any RDF sample when 25g of each were tested. Enumeration of E.coli and Campylobacter revealed <10 CFU per g/ml of every RDF tested.

Table 3. Fertilisers tested for the pathogens *E.coli*, *Campylobacter*, *Salmonella* and *Listeria* and their corresponding results.

Fertilizer	Possible coliforms present	High microbial load	*E.coli* CFU/g	*Campylo-bacter spp.* CFU/g	*Salmonella spp.* present	*Listeria spp.* present
Control (sterile water)			-	-	-	-
Triple super phosphate (TSP)			-	-	-	-
Calcium ammonium nitrate (CAN)			-	-	-	-
Struvite (sewage sludge processing)	✓		<10	<10	n.d.	n.d.
Struvite (potato processing)			-	-	-	-
Ash (poultry litter)			<10	<10	n.d.	n.d.
Ash (sewage sludge)	✓		<10	<10	n.d.	n.d.
Bed Ash (sewage sludge and green cuttings)			-	-	-	-
Ammonium nitrate	✓		<10	<10	n.d.	n.d.
Ammonium sulphate	✓		<10	<10	n.d.	n.d.
Ammonia water	✓		<10	<10	n.d.	n.d.
Pig urine	✓		<10	<10	n.d.	n.d.
Liquid concentrate	✓	✓	<10	<10	n.d.	n.d.
Blend 1	✓	✓	<10	<10	n.d.	n.d.
Blend 2		✓	<10	<10	n.d.	n.d.
Compost (L30 = 30% pig slurry)		✓	<10	<10	n.d.	n.d.
Compost (L70 = 70% pig slurry)		✓	<10	<10	n.d.	n.d.
Compost (L100 = 100% pig slurry)		✓	<10	<10	n.d.	n.d.

*n.d. = none detected

3.4. Presence of weeds

Pot trials testing for the presence of weeds in RDFs resulted in no growth of weeds in any replicates of the compost fertilized with RDFs, indicating that no viable weed seeds were present among any RDF products.

4. Conclusion

Investigations on the microbial load RDF products revealed that some types of RDFs have a much higher concentration of bacteria present, particularly liquid concentrate, liquid concentrate blends and compost products, as compared to drier products such as ashes and struvite. Fungal populations were scarce among most products, with one compost product being the exception. While the results of the tests for coliforms were inconclusive, the problematic scenario of the presence of the pathogenic bacteria *Salmonella spp., Listeria spp., E.coli* and *Campylobacter spp.* in suspected RDFs was ruled out. Therefore, it was concluded that the products are in accordance with the EU Fertilizing Products Regulation 2019/1009 and safe for use as fertilizers from a microbiological perspective. Furthermore, pot trials investigating the presence of potential weeds confirmed their absence in all RDF products.

5. References

Sahlstrom, L., 2003. A review of survival of pathogenic bacteria in organic waste. *Bioresource Technology,* 81(2), pp. 161-166.

Regulation of the European Parliament and of the Council laying down rules on the making available on the market of EU fertilising products [2019] OJ 2 170. Available at https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=OJ:L:2019:170:FULL&from=EN.

6. Appendix

Agrihealth E.coli and Campylobacter Result

Agrihealth Salmonella and Listeria Results.pc