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Escuela de Ciencias Biológicas e Ingeniería

Biological and Chemical analysis of sludge from the wastewater treatment system in a mining industry camp

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga

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DEDICATORIA

A mi hermano Maximiliano, que me recuerda cada día lo bonito de la vida, esperando poder ser una guía en su camino.

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RESUMEN

Los análisis químicos y biológicos son parte esencial de las plantas de aguas residuales. En un campamento minero tenemos que tener en cuenta no solo la contaminación que produce la industria, sino también nuestros propios desechos pueden representar una problemática ambiental. Las aguas residuales representan hoy en día una problemática socioambiental. Sin embargo, el tratamiento de las mismas puede proporcionarnos soluciones inmediatas. Para poder comprobar si estas plantas de tratamiento de aguas residuales están funcionando de manera correcta realizaremos diferentes tipos de ensayos. Es así como tenemos, para empezar, un análisis del código de clasificación de las características que contienen los residuos peligrosos y que significan: corrosivo, reactivo, explosivo, tóxico, inflamable y biológico infeccioso (CRETIB). Con esto podremos saber si nuestras muestras se considerarían riesgosos.

Para empezar con los ensayos, hicimos pruebas de pH y oxígeno disuelto, los cuales funcionan como bioindicadores, dentro de la PTAR. Luego pasamos a test de germinación con los lodos de PTAR, tanto en cajas Petri como en germinadores de plásticos. De esta manera se pudo comprobar si presentan inhibición en el crecimiento. Otro bioindicador fue el crecimiento bacteriano, utilizando diluciones seriadas en medio TSB, y sembrándolas en medios de LB agar.

Por último, tomando en cuenta que la resistencia antibiótica representa uno de los problemas más grandes que enfrenta la salud humana y animal. Se realizó una extracción de ADN de los lodos de aguas residuales, donde se tenía como interés secuenciar y encontrar los genes de resistencia antibióticos que tenemos en nuestras muestras. Con una amplia literatura podremos identificar los genes de resistencia que esperamos encontrar en nuestras muestras, y también las posibles soluciones.

Palabras clave: CRETIB, aguas residuales, PTAR, ADN.

ABSTRACT

Chemical and biological analyzes are an essential part of wastewater plants. In a mining camp, we have to consider that the pollution produced by the industry and our waste can represent an environmental problem. Wastewater represents today a socio-environmental problem. However, their treatment can provide us with immediate solutions. In order to check if these wastewater treatment plants are working correctly, we will conduct different tests. This is how we have, to begin with, an analysis of the classification code of the characteristics that hazardous waste contains and what it means: corrosive, reactive, explosive, toxic, flammable, and biologically infectious (CRETIB). With this, we knew if our samples would be considered risky.

To start with the tests, we made a pH and dissolved oxygen tests, which work as bioindicators inside the WWTP. Then we moved on to the germination test with the WWTP sludge in Petri dishes and plastic germinators. So, we can check if they show growth inhibition. We will also have bacterial growth as a bioindicator, using serial dilutions in TSB and seeding them in LB agar media.

Finally, considering that antibiotic resistance represents one of the biggest problems facing human and animal health. We will have a DNA extraction from the sewage sludge, where the interest was to sequence and find the antibiotic-resistance genes in our samples. With a vast literature, we will be able to identify the resistance genes we expect to find in our samples and the possible solutions.

Keywords: CRETIB, wastewater, WWTP, DNA.

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1. INTRODUCTION

Efficient water management in the mining industry is essential to guarantee the environmental and economic sustainability of the activity. One of the main challenges in this area is the treatment of wastewater generated in mining camps, which can contain a wide range of organic and inorganic contaminants.

Among the different wastewater treatment methods, the removal of solids through activated sludge systems has become one of the most widely used techniques. However, the analysis of the sludge generated by these systems is essential to assess its quality and determine if it is suitable for final disposal.

In this context, the objective of this study is to carry out a biological and chemical analysis of the sludge generated by the wastewater treatment system in a mining camp, in order to evaluate its quality and determine if it complies with current environmental and sanitary regulations. To do this, microbiological and chemical analyzes of the sludge will be carried out, evaluating parameters such as germination test, the presence of microorganisms, DNA extraction, and other indicators of water quality.

The results obtained in this study may be useful to improve water management in the mining industry and guarantee its long-term sustainability.

2. JUSTIFICATION

Today the need to minimize waste, as well as its proper and safe disposal, is of paramount importance worldwide. This has led to the search for technological alternatives and changes in management policies to generate non-hazardous and stable waste for proper disposal or reuse.

Sludge from a mining industry wastewater treatment plant (WWTP) is generated considerably, causing pollution problems. However, these may be favorable substrates for organic fertilizers.

The selection of some process for stabilizing a particular sludge depends on several factors, such as the quantity and quality of sludge to be treated, the particular conditions of the site, and the financial situation in each case. In many countries, the use of sludge requires an expensive but justified purposes infrastructure, as it solves pollution problems and incorporates nutrients by

recycling vital elements in natural biological cycles, and converts hazardous waste into a non-hazardous and profitable resource.

3. GENERAL AND SPECIFIC OBJECTIVES

3.1 General Objective

Analyze sludge from wastewater plants using ecotoxicology bioassays to determine their toxicity in the environment.

3.2 Specific Objectives

- Evaluate the bioindicators of wastewater purity during and after its treatment within the WWTPs.
- Evaluate the germination of radish and lettuce seeds subjected to different doses of sewage sludge.
- Identify the bacterial load of wastewater sludge.
- Extract DNA from sewage sludge for the sequencing process and identification of resistance genes

4. THEORETICAL BACKGROUND

4.1 CRETIB

The CRETIB code refers to the characteristics that make a waste considered hazardous; compliance with one or more of it makes a waste hazardous; likewise, non-hazardous waste mixed with hazardous waste is contaminated and becomes hazardous[1].

CRETIB, by their acronym in Spanish, Corrosivity (C), Reactivity (R), Explosiveness (E), Environmental toxicity (T), Flammability (I), Biological Infectious (B)

A waste is considered dangerous due to its corrosiveness when it has any of the following properties: In the liquid state or aqueous solution, it has a pH of 2.0 to 12.5. A temperature of 55 °C is capable of corroding carbon steel at a rate of 6.35 millimeters or more per year[1,2,3].

A waste is considered dangerous due to its reactivity when it has any of the following properties: Under normal conditions (25 °C and 1 atmosphere), it combines or polymerizes violently without detonation. When in contact with water in a ratio (waste-water) of 5:1, 5:3, 5:5, it reacts violently,

forming gases, vapors, or fumes. Under normal conditions, when contacted with pH solutions, acid (HCl 1.0 N) and basic (NaOH 1.0 N), in a ratio (residue-solution) of 5:1, 5:3, 5:5, react violently, forming gases, vapors, or smoke. It contains cyanides or sulfides that, when exposed to pH conditions between 2.0 and 12.5, can generate toxic gases, vapors, or fumes in quantities greater than 250 mg of HCN/kg of waste or 500 mg of H2S/kg of waste. It is capable of producing free radicals[1,2,3].

A waste is considered dangerous due to its explosiveness when it has any of the following properties: It has an explosiveness constant equal to or greater than that of dinitrobenzene. It can produce a detonating or explosive reaction or failure at 25°C and 1.03 kg/cm² of pressure. 1,2,3

A waste is considered dangerous due to its toxicity to the environment when it has the following property: When subjected to the extraction test for toxicity according to the official Mexican standard NOM-CRP-002-ECOL/1993[1,2,3].

A waste is considered dangerous due to its flammability when it has any of the following properties: In an aqueous solution, it contains more than 24% alcohol by volume. It is liquid and has a flash point below 60°C. It is not a liquid but is capable of causing fire due to friction, moisture absorption, or spontaneous chemical changes (at 25°C and 1.03 kg/cm^2). These are flammable compressed gases or oxidizing agents that stimulate combustion [1,2,3].

A waste with biological characteristics is considered dangerous when it has any of the following properties: When the waste contains bacteria, viruses, or other microorganisms capable of infection. When it contains substances produced by microorganisms that cause harmful effects to living beings, the mixture of hazardous waste and this standard with non-hazardous waste will be considered hazardous waste[1,2,3].

4.2 Germination Test

The germination test is carried out in the laboratory, which consists of placing seeds in a moist substrate in controlled temperature, humidity, and light conditions. Hence, they germinate and reach a development level; in this way, the essential structures of the plant can be evaluated and determine if they are suitable to continue with their average growth[4].

The germination test evaluates the percentage of seeds in a batch with the ability to germinate and produce normal seedlings under ideal environmental conditions (temperature, humidity, and aeration) for the germination process to obtain information on the quality of the different batches

for its marketing.[4,5]

It is essential to highlight that, during the germination period and the first days of seedling development, numerous physiological processes occur in which the presence of a toxic substance can interfere with the survival and normal development of the plant. For this reason, germination is a stage of great sensitivity to adverse external factors.[6,7]

Toxicity tests based on seed germination and root elongation can be carried out with various species, including economically important plants that are easily accessible and germinate and grow rapidly. The application of the test is usually carried out as a toxicity test or through a battery of tests with other bioindicators.[8]

4.3 Assays In Vitro

A culture medium is a set of nutrients, growth factors, and other components that create the necessary conditions for the development of microorganisms. In microbiology, sowing means inoculating or introducing a sample, such as river water, in a particular culture medium. This medium stimulates the growth of the bacteria in the sample and thus obtains visible colonies[9]. Although the bacteria cannot be seen, what can be seen are the colonies that form when billions of bacteria in these culture media have all the necessary characteristics for their mass replication. Each bacterial species gives rise to colonies with unique properties, so these culture media allow us to know or make an approximation of which bacteria, or bacteria, are in our sample[9,10].

Culture Media for Microbiology presents conditions such as: having nutritional substances such as amino acids, carbohydrates, polyalcohol, vitamins, and minerals. In addition, a pH that allows optimal development and is previously sterilized or prepared in aseptic conditions is necessary for the growth of microorganisms. This mixture of nutrients, in adequate concentrations and optimal physical conditions, allows the growth of microorganisms (fungi, bacteria, and others)[10].

The metabolic diversity of these is so great that the variety of culture media is enormous, and there is no universal culture medium suitable for all of them. One of the most common is general enrichment media that favor the growth of some microorganisms. They allow increasing the number of microorganisms to be able to recognize the bacterial growth of the sample. For their part, we can use AGAR, which is used as a gelling agent to give solidity to culture media. In bacteriological agar, the dominant component is a polysaccharide that is obtained from certain

marine algae. It has the undoubted advantage that, with the exception of some marine microorganisms, it is not used as a nutrient. A 1-2% agar gel liquefies around 100ºC and gels around 40ºC, depending on its degree of purity[11].

On the other hand, we have the liquid medium LURIA BROTH (Luria Bertani LB), or LB broth contains casein peptone and yeast extract that provide the medium with the necessary nutrients for the optimal development of most microorganisms. In addition, sodium chloride helps maintain osmotic balance[12].

4.4 Wastewater

Water is a vital resource for humans, not only for consumption but for all industrial processes and production. Unfortunately, our activity alters its natural state and pollutes it, causing severe environmental damage to rivers and seas and making it impossible to use for agriculture[12]. That is why ensuring proper water treatment for reuse and return to the environment is essential. Wastewater treatment consists of a series of physical, chemical, and biological processes that remove water contaminants so humans can use them[12,13].

The treatment of wastewater is a practice that, although it has been carried out since ancient times, today is essential to maintain our quality of life. There are many techniques for each type of wastewater treatment, with a long tradition that has been improved over the years in the knowledge and design of WWTPs[13].

The increasingly demanding water treatment regulation laws that must be complied with have paved the way for applying new water treatment technologies. Many of these technologies allow the recovery of resources and give significant value to the waste generated, such as the reuse of wastewater for irrigation, recovery of oils and fats from wastewater, or power generation[14].

We can find three types of wastewater treatment:

Physical Treatments

They are those methods in which a physical separation is applied, generally of solids. These methods usually depend on the physical properties of the contaminants, such as viscosity, particle size, and buoyancy. Among them, we can find sieving, precipitation, separation, and filtration of solids[15].

Chemical Treatments

These methods depend on the chemical properties of the contaminant or reagent incorporated into the water. We can highlight the elimination of iron and oxygen, the elimination of phosphates and nitrates, coagulation, electrochemical processes, oxidation, and ion exchange[15].

Biological Treatments

Biological processes are used in these methods in such a way that it is intended to eliminate colloidal contaminants. They are microorganisms that act on the matter in suspension, transforming it into sedimented solids. They can be aerobic or anaerobic processes, such as activated sludge, trickling filters, anaerobic digestion, or lagoons[15].

Wastewater content

Wastewater is also known as black water. All these terms refer to the water obtained after the human intervention, which alters its natural composition due to organic and chemical waste.

Wastewater generally contains organic matter, plant residues, animals, fats, and oils, among other elements. However, it also has inorganic matter that can be small or large solids such as fabrics, plastics, chemicals, and sand, among other compounds[15,16].

For its part, bacteria are the microbiological components of wastewater that must be considered when passing through a treatment plant. These usually come mainly from feces, fungi generated from industrial waters, and protozoa and actinomycetes[16,17,19].

However, we also have greywater. Gray water is produced after being used by humans in their daily lives. They usually come from shower drains, bathtubs, washing machines, and dishwashers. That is, they are those that generally contain soap and small solid residues. Among its characteristics, it stands out that they have a much lower level of fecal content and differ from wastewater because they have a lower pollution load. This makes them ideal for recycling[15,16,18,80].

4.4.1 Characteristics of the wastewater

We can define wastewater quality by its chemical, physical, and biological characteristics. Physical parameters include odor, color, temperature, suspended and dissolved solids or organic and inorganic fractions, turbidity, oil, and fat. Chemical parameters associated with the organic content of wastewater include total organic carbon demand (TOC), chemical oxygen demand (COD), biochemical oxygen demand (BOD), and total oxygen demand (TOD). Inorganic chemical parameters include hardness, salinity, pH, alkalinity, acidity, iron, chlorides, manganese, sulfates, sulfides, heavy metals such as lead, mercury, zinc, copper, and chromium, organic nitrogen, nitrite and nitrate, ammonia, and phosphorous. Bacteriological parameters include fecal coliforms, coliforms, specific pathogens, and viruses[14,15,17].

Typical elements found in untreated domestic water	
Minerals	Microorganisms
Anions Bicarbonate (HCO;) Carbonate (CO,) Chloride (CI) Nitrate (NO3) Phosphate (PO4) Sulfate (SO4) Cations Calcium (Ca) Magnesium (Mg) Potassium (K) Sodium (Na) Other constituents Aluminum (Al) Boron (B) Fluoride (F) Manganese (Mn) Silica (SiOz) Total alkalinity (as CaCO;) TDS	Total coliform Fecal coliform Fecal streptococci Enterococci Shigella Salmonella Pseudomonas aeroginosa Clostridium perfringens Mycobacterium tuberculosis Protozoan cysts Giardia cysts Cryptosporidium cysts Helminth ova Enteric virus

Table 1: Typical elements found in wastewater [15]

For wastewater treatment facilities, the characteristics of the wastewater are considered. Table 1 shows us the typical minerals and microorganisms found in domestic waters.[15]

4.4.2 Wastewater reuse

The reuse of treated water has different regulations in different countries of the world. For example, in some countries, its uses may be for supply. However, it is only allowed for industrial or agricultural uses in others. Nevertheless, water reuse regulation has something in standard worldwide: it must meet demanding quality criteria.[16,17]

In Ecuador, approximately 12% of domestic wastewater is treated, leaving a large 88% without treatment that is channeled directly to streams and rivers. According to data from the National Water Secretariat, SENAGUA, all the rivers in Ecuador below 2,800 masl are severely polluted and their water is unfit for human consumption. Faced with this reality, there are projects to build large wastewater treatment plants, such as Vindobona. This is a new project to build a huge plant of wastewater treatment for the city of Quito, but due to the high cost of the project (900 million dollars) it has not started yet[82].

The quality criteria of reclaimed water vary according to its subsequent uses, which are also detailed in the laws of each country. Currently, reclaimed water can be used, for example, for irrigation of urban green areas, street cleaning, fire-fighting systems, industrial vehicle washing, agricultural irrigation, refrigeration and industrial evaporative condensers, water from various industrial processes, and recharge of aquifers. However, its use is prohibited for human consumption in hospitals and health facilities, swimming pools or fountains, and ornaments in public spaces or interiors of public buildings.[16,18]

Finally, once the solid waste is extracted from the wastewater, it can be used as compost for future use in agriculture. Both solid waste and adequately treated wastewater do not represent a risk to flora and fauna of the environment.[19,20]

4.5 Antibiotic Resistance

The use, and overuse, of antibiotics have had a severe environmental impact that has received increasing scientific interest in recent years. One of the main entrances to the environment of these drugs is wastewater since between 40 and 90% of antibiotics are not metabolized through biochemical routes and are excreted through urine or feces, both in humans and in animals arriving at the wastewater treatment stations (WWTPs). In addition, these facilities have conventional treatments that are not explicitly designed for eliminating drugs, so they can act as sources of dissemination since antibiotics or their transformation products enter the environment through the discharge of their effluents.[21,22]

On the other hand, due to the increased use of antibiotics, specific pathogens have developed resistance mechanisms that inhibit the actions aimed at eliminating them, known as ARB

(antibiotic resistance bacteria). Antibiotic resistance is recognized as one of the most critical challenges of contemporary medicine and public health since the chances of success in clinical treatments are reduced.[21,22]

Studying the bacteria in water treatment plants has become a fundamental task in monitoring resistance to antibiotics in the environment. An international team of scientists has compared the number of antibiotic-resistance genes in different water treatment plants in seven European countries: Finland, Norway, Germany, Ireland, Spain, Portugal, and Cyprus.[21,23]

The researchers analyzed the antibiotic-resistance genes in the bacteria that reach the wastewater-regenerating plants and those that survive after the water purification.[79]

The study results indicate that wastewater treatment plants effectively eliminate antibiotic-resistant bacteria. However, the study also notes that a treatment plant can function as an incubator for antibiotic resistance under certain conditions.[24,25]

As previously indicated, specific concentrations of antibiotics can reach WWTPs through urban wastewater from hospitals, residential areas, factories, and the livestock industry. Therefore, these waters are also susceptible to antibiotic Resistant Bacteria (ARBs), and Genes (ARGs) since they also reach wastewater through their excretion by humans or animals. WWTPs are thus important reservoirs not only for the spread of antibiotics but also for resistance, threatening the ecological balance and the safety of the receiving environments if this type of water is not adequately treated[22,26].

There are different classification systems for antibiotics, depending on the nature of their origin (natural or synthetic) origin, according to their chemical structure, activity spectrum, or inhibition mechanism[23,26].

Regarding the inhibition mechanism, the following groups of antibiotics are distinguished according to the objective of action[27]:

- Cell wall synthesis.
- DNA gyrase
- Metabolic enzymes
- RNA polymerase – DNA dependent
- Protein synthesis.

From a genetic perspective, bacterial resistance to antimicrobials can be intrinsic or acquired. Intrinsic resistance is a consequence of genes inherent to the microorganism, which are found

naturally on the bacterial chromosome and determine structural or physiological characteristics that confer resistance to antimicrobials. This would be the case of resistance to some beta-lactams due to the production of AmpC in enterobacteria or other gram-negative or multi-resistance due to the presence of expulsion pumps. This type of resistance is the least worrying from a cynical point of view because it is predictable[27,28,80].

Acquired resistance occurs in microorganisms that were initially sensitive to an antimicrobial and that have been able to develop resistance due to spontaneous mutations or by the acquisition of resistance genes from other microorganisms and is what constitutes a problem[27.29].

4.6 DNA Extraction

Currently, the study of genetic variation between individuals, populations, and species to explain ecological-evolutionary patterns and processes is approached through molecular markers, DNA segments with or without known function that provide information on allelic variation and allow individuals to be distinguished[30,31]. These markers are obtained with techniques such as PCR (Polymerase Chain Reaction) and sequencing, which make it possible to analyze the variation in the DNA molecule with unprecedented detail[31].

Molecular data have made it possible to study with greater precision the patterns of genetic diversity and its distribution; behavior; natural selection; biological interactions; the composition, functioning, and dynamics of microbial communities; phylogenetic relationships, among others. However, the application of molecular techniques begins with DNA extraction, and the successful obtaining of reliable and reproducible data depends, to a large extent, on the extraction of whole and pure DNA[31,32,33].

The extraction consists of the isolation and purification of DNA molecules and is based on the physicochemical characteristics of the molecule. DNA comprises two chains of nucleotides linked together to form a double helix. Nucleotides comprise a sugar (desoxyribose), a phosphate group, and a nitrogenous base (adenine, guanine, thymine, or cytosine). The union of the nucleotides occurs between the phosphate group and the sugar through phosphodiester bonds, giving rise to the skeleton of the molecule. The bases of opposite chains are joined by hydrogen bonds and stabilize the helical structure[31,35].

Phosphate groups are negatively charged and polar, giving DNA a net negative charge and making it highly polar, characteristics that are used for extraction. In addition, phosphate groups tend to repel each other due to their negative charge, which allows DNA to dissolve in aqueous solutions and form a moisturizing layer around the molecule. However, in the presence of ethanol, the moisturizing layer breaks down, and the phosphate groups are exposed. Under these conditions, binding with cations such as Na+ is favored, which reduces the repulsive forces between the nucleotide chains and allows the DNA to precipitate. On the other hand, the net negative charge of DNA allows it to bind to positively charged inorganic molecules and matrices[36,37].

4.7 Bioindicators

4.7.1 Ph Bioindicator

pH is a measure that indicates the acidity of water. The range varies from 0 to 14, with 7 being the average range (neutral range). A lower pH indicates acidity, while a pH greater than 7 indicates that it is more alkaline.[38,39]

pH measures the relative amount of hydrogen and hydroxide ions in water. If the water contains more hydrogen ions, it has a higher acidity, while water containing more hydroxide ions indicates a basic range.[38,40]

If the sample has a pH less than 5.5 or greater than 8.6, the water is very acidic, and fish and other organisms will not be able to survive. It is acceptable if this is between 5.5-5.9 or 8.1-8.5; there are certain organisms that can withstand that acidity but not many[39]. The acidity would be good if we have a result of 6.0-6.4 or 7.6-8.0. An excellent pH is one that we find between 6.5 and 7.5.[41]

4.7.2 Dissolved Oxygen

The oxygen meter measures the amount of dissolved oxygen in liquids. Two measurement scales are commonly used: parts per million (ppm); or saturation percentage (%), defined as the percentage of dissolved oxygen in 1 liter of water, concerning the maximum amount of dissolved oxygen that 1 liter of water can contain. It is necessary to determine the amount of dissolved oxygen in the water because it is an indicator of the quality of the water. Thus it is essential, for example, to control urban and industrial wastewater where low concentrations of this parameter are a sign of contamination. [42,43,44]

5. MATERIALS AND METHODS

5.1 Drying WWTP

In order to have greater precision in the dilutions of the tests, dried sludge from WWTPs is needed. For this reason, drying beds were created, which were made with cardboard and aluminum. First, the samples were extracted from the aeration of the WWTPs, both from the black and gray waters and the mixed ones. After this, we wait for the sludge to settle to remove as much water as possible. After this, they were placed on aluminum beds near the heater to dry more quickly. Once this dry sludge was obtained, the corresponding experiments were carried out.

5.2 Measured Parameters Of The WWTP

In the case of the WWTP, to obtain the concentration of hydrogen ions, samples were taken every three days from the sludge undergoing aeration and from the final discharge wastewater from both fields, and take ph measurements. The case of "A camp" has a plant for gray water and one for black water, unlike "B camp," which has a plant mixed with black and gray water. Likewise, the dissolved oxygen of the discharge water and the aerated sample was measured using the dissolved oxygen meter "HI 9146" previously calibrated. In addition, measurements were made every six days for the residual and total chlorine parameters.

5.3 Bacterial identification

Tryptic Soy Broth (TSB) is a nutrient medium that supports the growth of various microorganisms, especially facultative anaerobic bacteria, and common aerobic bacteria. TSB solution was used for bacterial identification of wastewater[45,46]. To prepare TSB, we suspend 30 grams of the medium in one liter of distilled water. Mix well and dissolve with heat and frequent agitation. Next, we proceed to boil for a minute until completely dissolved. Finally, we must sterilize in an autoclave at 121ºC for 15 minutes. Then in the laminar flow chamber, we place the necessary amount in each tube. The untreated wastewater was extracted into 4-liter bottles that entered the WWTP aeration to obtain the sample. Once settled, 1 ml of residual water placed in 9 ml of medium was taken. We obtained a 1:10 ratio and proceeded to make a serial dilution up to 1:100,000. The type of bacterial

growth in both tubes was monitored at 24 and 48 hours.

Five types of bacteria could be found in bacterial growth with wastewater in the TSB medium. Strictly aerobic bacteria are part of an organism that needs an environment containing diatomic oxygen (a gas made up of two oxygen atoms) to exist and develop properly. That is, these bacteria need oxygen for cellular respiration.[46] In addition, they use oxygen to oxidize substrates (such as fats and sugars) for energy. On the other hand, there are facultative bacteria that are adapted to grow and metabolize both in the presence and absence of oxygen. We also have aerotolerant bacteria that can survive in the presence of oxygen but do not use it as they are anaerobic. They tolerate low concentrations of oxygen: 2 to 8%. Strictly anaerobic bacteria need an atmosphere without oxygen since this element is toxic to them to grow on the surface of a culture medium. They lack the cytochrome oxidase, superoxide dismutase, and catalase system to metabolize O2. Therefore, they require a low redox potential.[47] They are widely distributed in nature. They live in the normal flora of mucous membranes, skin, mouth, upper airways, and the female genitourinary tract and intestine. Furthermore, microaerophilic bacteria require oxygen at levels lower than those present in the atmosphere. Therefore, they use oxygen but in meager amounts.[48]

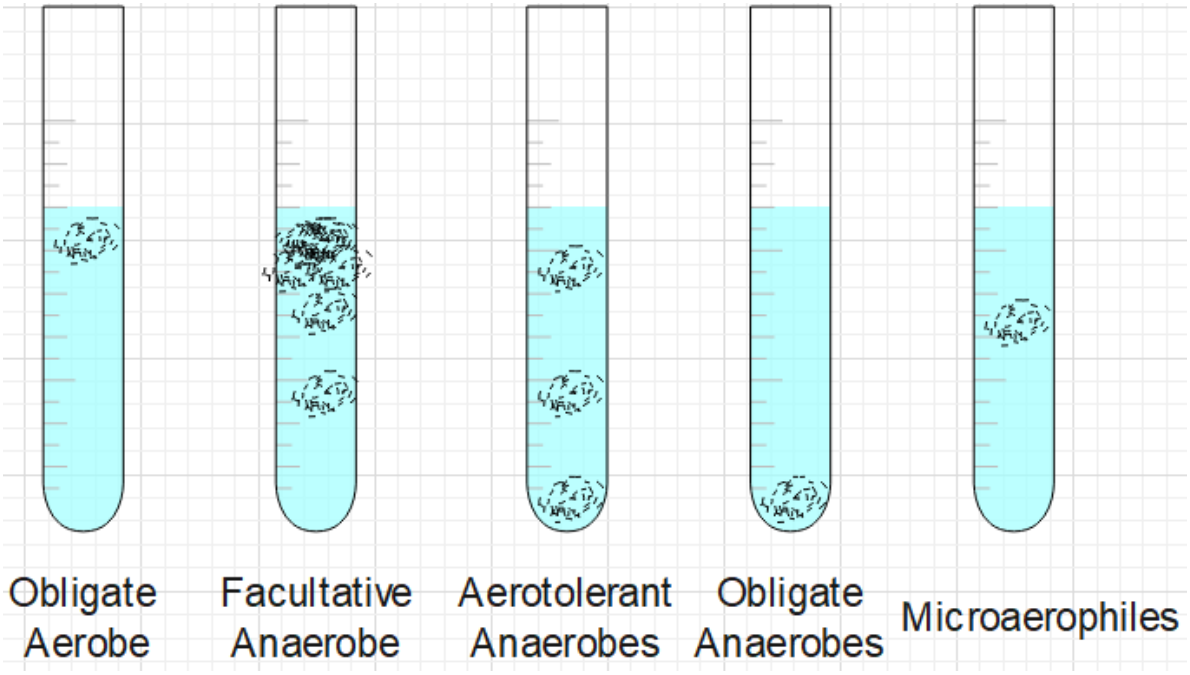

Figure 1: Types of bacterial growth in test tubes according to oxygen requirements.[48]

5.3.1 Bacterial Medium

LURIA BROTH (Luria Bertani LB) or LB broth contains casein peptone and yeast extract that provide the medium with the necessary nutrients for the optimal development of most microorganisms.[49] In addition, sodium chloride helps maintain osmotic balance. For the preparation, we rehydrated 20 g of the medium in one liter of distilled water. It is then heated with frequent stirring until the complete dissolution of the lumps. Next, we sterilize in an autoclave at 121ºC (15 lbs of pressure) for 15-30 minutes. Once we finished, lb agar was distributed to the previously sterilized Petri dishes. Finally, we seal the boxes with parafilm and keep them in an environment of 4 ºC to 8 ºC for future uses.[50]

5.4 Germination in Petri using WWTP Sludges.

Firstly, dilutions were made for each WWTP 1:150 (sludge: soil) with 298 grams of soil and 2 grams of sewage sludge. Therefore, the samples are taken from the gray water plant, the black water plant from A camp, and the mixed water sample (black and gray) from B camp. After these are dry, they are diluted in 300 ml of distilled water and stirred for 20 min. Then, 5 ml of the dilution is placed on absorbent towels inside the Petri dishes. Three replicates and two controls are made, obtaining three replicas of black water, 3 gray water, and three mixed waters. Subsequently, 25 radish seeds were placed in each Petri dish, and their growth was monitored for three days. After that, the process was repeated with radish and with lettuce seeds seven times.

5.5 Variables evaluated for germination

Emergence speed index (ESI): it is obtained through the daily count of the seedlings that emerged from sowing, taking as emerged seedlings those that protrude from the substrate. The ESI emergency velocity index is calculated using the expression proposed by Maguire in 1962[51]

$$ESI = \sum_{i=1}^{n} \frac{X_i}{N_i} \qquad \textbf{(1)}$$

Where:

ESI = emergency speed index; *Xi*=Number of seedlings that emerged per day; *Ni*= Number of days after sowing; *n*= Number of counts.

At the end of the study, the total emergence percentage (TPE) can be obtained, which consists of counting each seedling that emerged until the last day of the evaluation. Then, the result is obtained by dividing the number of emerged seedlings by the Number of seeds sown and multiplying by one hundred.[52]

$$TPE = \frac{E}{P} * 100 \qquad \textbf{(2)}$$

Where:

TPE= Total percentage of emergence; *E*= Seeds that emerged on the last day of counting;

P= Total seeds that were used in the germination test or planted.

5.6 Germination and Development of Seedlings in Plastic Germinators using WWTP Sludge

The seedlings were developed in two plastic seedbeds with divided columns in the presence of WWTP sludge. One for the radish and one for the lettuce. The first column of both nurseries was the control, where only soil from the pots of the university was placed. The second is for a dry dilution with 70% regular soil, 20% vermi compost, and 10 % WWTP sludge. The third mixture was dilution with 70% regular soil, 20% organic compost, and 10 % WWTP sludge. In the fourth parcel we add dilution with 70% regular soil, 10% vermi compost, 10% organic compost and 10 % WWTP sludge. Finally, in the last one we add dilution with 70% regular soil, 20% vermi compost, and 10 % organic compost. Ten repetitions of each dilution were performed. After this, holes were made in each column where three radish seeds were placed in the first seedbeds and three lettuce seeds in the second plastic seedbeds. Finally, the seedlings were irrigated and monitored for 15 days to observe the presence of abnormalities or inhibition of seedling growth due to WWTP sludge.

5.7 DNA Extraction

For the extraction, was use a PureLink Microbiome DNA purification kit from ThermoFisher. To prepare the lysate, the sample is centrifuged at 14,000 × g for 10 minutes to pellet the organisms. Moreover, we carefully removed the supernatant and discarded it. Resuspend the microbial pellet in 800 µL of S1—Lysis buffer, pipet up and down or vortex to resuspend, then transfer the sample to the bead tube. Add 100 µL of S2—Lysis Enhancer, and briefly vortex. We incubated at 65°C for 10 minutes. To go on to homogenize by beating the beads for 10 minutes at maximum speed in the vortex mixer. Centrifuge at 14,000 × g for 2 minutes. Transfer up to 500 µL of the supernatant to a clean microcentrifuge tube, avoiding bead sediment and debris.

To bind the DNA to the column. Add 900 µL of S4—Binding buffer and vortex briefly. Load 700 µL of the sample mixture into a spin tube and spin column assembly, centrifuge at 14,000 × g for 1 minute, and repeat the latter process.

Finally, to wash and elute the DNA, the spin column is placed in a clean collection tube, and 500 µL of S5 is added: we wash the buffer and then centrifuge the column-spin tube assembly at 14,000 × g for 1 minute. We discarded the flow-through and then centrifuged the spin column and tube assembly at 14,000 × g to 30 seconds. The spin column is placed in a clean tube. We add 100 µL of S6: elution solution and then incubate at room temperature for 1 minute. Lastly, centrifuge the spin tube and column assembly at 14,000 × g for 1 minute and discard the column. With this, we obtain the purified DNA in the tube.

6. RESULTS

6.1 Measured Parameters of the WWTP

6.1.1 pH Measured

As shown in Table 2, very low values, such as 4.01, are shown in the first pH measurement. These values indicate a very acidic pH for the water that goes into the environment, which is not recommended. However, we see a noticeable improvement from the next shot. Thus, values improve and get an average very close to neutral, which is ideal for it to be released into the environment. Therefore, it is considered acceptable that the treated water has a pH between 1.5 to 8.5, with 7 being the expected measurement. Having a pH of 6.5 to 7.5 means that we find an excellent result, which is as expected [40,54].

6.1.2 Dissolved oxygen

6.1.2.1 Dissolved oxygen (ppm)

As we know, free oxygen is essential for the life of fish, plants, algae, and other organisms. As shown in Table 2, we have an average of 4.2 to 4.79. If we had a 5 to 6 ppm concentration, it means there is enough oxygen for most species. However, in a few shots, we can find these values.
We also have less than three ppm values, which harms the ecosystem. From this point downward, the ecosystem experiences hypoxia. This point should not be reached because values below two ppm are fatal for most species because the ecosystem suffers from anoxia. Although the values are suitable for irrigation, they should be improved so there are fewer environmental problems[43].

6.1.2.2 Percent of dissolved oxygen

We found average values from 62.46% to 69.02% dissolved oxygen in the water, which indicates an appropriate value for wastewater. We will need a higher percentage if this is to house marine species. However, these values are considered acceptable for irrigation and show little environmental risk [44].

	MIXTURE WWTP		GRAY WWPT		BLACK WWTP	
	Final Discharge	Oxigenator/ Sludges	Final Discharge	Oxigenator/ Sludges	Final Discharge	Oxigenator/ Sludges
	JULY 23 2021					
pH	4.29	4.12	6.53	5.93	4.01	4.11
DISSOLVED OXYGEN/ppm	4.53	2.61	4.36	3.18	4.61	3.3
DISSOLVED OXYGEN/ %	73.8	43.2	74.6	60.22	69.9	50.4
	JULY 31 2021					
pH	7.2	6.9	7	7.1	7.2	6.9
DISSOLVED OXYGEN/ppm	5.4	4.32	3.2	4.36	4.39	3.41
DISSOLVED OXYGEN/ %	69.3	78.5	70	68.3	69.5	50.4
	AUGUST 3 2021					
pH	7.12	7	6.98	7.1	6.95	7
DISSOLVED OXYGEN/ppm	4.81	4.9	5.1	4.31	5.12	5.15
DISSOLVED OXYGEN/ %	69.8	70.5	71.6	69.9	70.9	69.7
	AUGUST 6 2021					
pH	6.97	7.1	7.11	6.75	6.9	7
DISSOLVED OXYGEN/ppm	4.78	4.85	3.78	4.85	4.78	4.89
DISSOLVED OXYGEN/ %	64.7	68.3	61.4	72	71	73.1
	AUGUST 8, 2021					
pH	6.87	7.01	7.01	6.97	6.87	7.11
DISSOLVED OXYGEN/ppm	4.44	4.69	4.56	4.72	4.81	4.78
DISSOLVED OXYGEN/ %	63.9	67.8	67.71	65.15	64.8	68.7
	AVERAGE					
pH	6.49	6.426	6.926	6.77	6.386	6.424
DISSOLVED OXYGEN/ppm	4.792	4.274	4.2	4.284	4.742	4.306
DISSOLVED OXYGEN/ %	68.3	65.66	69.062	67.114	69.22	62.46

Table 2. pH values, dissolved oxygen in ppm, and percentage of dissolved oxygen during four measurements. The values of mixed, gray, and black water from the WWTP were taken. The final discharge and oxygenator/sludge samples were taken from each WWTP. Finally, we have an average of each value

6.2 Germination Test in Petri

6.2.1 Radish Germination

As we can see in Figure 2 and Table 3, we realize that the seeds have almost constant growth regardless of their content. The emergency speed does not show a more significant difference between the controls and those containing WWTP. We also have a percentage of emergence that does not show us any inhibition of growth on the part of the sewage sludge.

Although we can notice a slight difference in the emergency speed from one experiment to another,

this may be due to environmental factors since they all grow at almost the same rate within each experiment.

RADISH																				
	EXPERIMENT 1					EXPERIMENT 2					EXPERIMENT 3					EXPERIMENT 4				
			Plantulas					Plantulas					Plantulas					Plantulas		
	ESI	TPE	Day 1	Day 2	Day 3	ESI	TPE	Day 1	Day 2	Day 3	ESI	TPE	Day 1	Day 2	Day 3	ESI	TPE	Day 1	Day 2	Day 3
Control 1	19.5	96%	0	23	24	34.8333333	100%	14	25	25	34	96%	14	24	24	36.66666667	92%	18	22	23
Control 2	19.83333333	100%	0	23	25	34.8333333	100%	14	25	25	37.16666667	92%	18	23	23	36	96%	17	22	24
WWTP A 1	19	96%	0	22	24	31.8333333	100%	12	23	25	35	96%	15	24	24	33	96%	13	24	24
WWTP A 2	20.83333333	100%	0	25	25	33.3333333	100%	13	24	25	35.33333333	100%	15	24	25	35.83333333	100%	15	25	25
WWTP A 3	20.83333333	100%	0	25	25	30.8333333	100%	11	23	25	33.83333333	100%	13	25	25	36	96%	16	24	24
WWTP B 1	19.16666667	92%	0	23	23	33.3333333	100%	13	24	25	31	96%	12	22	24	35.16666667	92%	16	23	23
WWTP B 2	14	72%	0	16	18	30.8333333	100%	10	25	25	33.16666667	92%	14	23	23	38.16666667	92%	19	23	23
WWTP B 3	15.83333333	76%	0	19	19	28.8333333	100%	8	25	25	35.66666667	92%	17	22	23	39.66666667	92%	21	22	23
	EXPERIMENT 5					EXPERIMENT 6					EXPERIMENT 7									
Control 1	13.33333333	88%	0	12	22	11	84%	0	8	21	7	60%	0	4	15					
Control 2	12.66666667	92%	0	10	23	14.1666667	92%	0	13	23	10.16666667	68%	0	9	17					
WWTP A 1	14.83333333	88%	0	15	22	13.8333333	76%	0	15	19	11.16666667	68%	0	11	17					
WWTP A 2	15.83333333	100%	0	15	25	14	96%	0	12	24	9.5	72%	0	7	18					
WWTP A 3	13.16666667	92%	0	11	23	12.3333333	88%	0	10	22	9.166666667	68%	0	7	17					
WWTP B 1	15.5	96%	0	15	24	14.6666667	80%	0	16	20	13.66666667	80%	0	14	20					
WWTP B 2	13.33333333	76%	0	14	19	12.6666667	80%	0	12	20	11.66666667	80%	0	10	20					
WWTP B 3	14.33333333	88%	0	14	22	11.3333333	76%	0	10	19	11.33333333	76%	0	10	19					
WWTP GRIS	13.33333333	76%	0	14	19	11.8333333	88%	0	9	22	14.66666667	92%	0	14	23					
WWTP GRIS	11.16666667	80%	0	9	20	13.1666667	80%	0	13	20	14	84%	0	14	21					
WWTP GRIS	16.5	96%	0	17	24	10.8333333	88%	0	7	22	9.333333333	64%	0	8	16					

Table 3. Radish Germination in petri dishes. The results correspond to two controls with distillated water and two with mixture WWTP, two with black WWTP, and two with gray WWTP sludges. We have the germination for the first, second and third day. Finally, we have the emergency speed index and total percentage of emergence.

Fig 2. Radish Germination, third day. These experiments correspond to two controls with distillated water and two with mixture WWTP, two with black WWTP, and two with gray WWTP sludges.

6.2.2 Lettuce Germination

Lettuce behaves similarly to radish germination. As we can see in Figure 3 and Table 4, we notice that the seeds have almost constant growth regardless of their content. The emergency speed does not show a more significant difference between the controls and those containing WWTP. Although we must mention differences, there are occasions where even the Petri dishes that contain WWTP have a higher emergency speed. On the other hand, the emergence percentage does not show any growth inhibition of part of the sewage sludge.

Although we can notice a slight difference in the emergency speed from one experiment to another. This may be due to environmental factors since, like the radish, they all grow at almost the same rate within each experiment.

LETTUCE															
	EXPERIMENT 1					EXPERIMENT 2					EXPERIMENT 3				
			Plantulas					Plantulas					Plantulas		
	ESI	TPE	Day 1	Day 2	Day 3	ESI	TPE	Day 1	Day 2	Day 3	ESI	TPE	Day 1	Day 2	Day 3
Control 1	39.33333333	100%	19	24	25	29.1666667	92%	10	23	23	37	96%	17	24	24
Control 2	33.83333333	100%	14	23	25	31.3333333	100%	11	24	25	35.33333333	100%	15	24	25
WWTP A 1	37.83333333	100%	17	25	25	31.8333333	100%	12	23	25	33.83333333	100%	14	23	25
WWTP A 2	39.33333333	100%	19	24	25	31.3333333	100%	11	24	25	36.83333333	100%	16	25	25
WWTP A 3	42.83333333	100%	22	25	25	27.6666667	92%	9	22	23	33.66666667	92%	15	22	23
WWTP B 1	44.83333333	100%	24	25	25	26.3333333	100%	7	22	25	37.5	96%	18	23	24
WWTP B 2	38.33333333	100%	18	24	25	27.5	96%	8	23	24	31.5	96%	12	23	24
WWTP B 3	37.83333333	100%	17	25	25	30.8333333	100%	10	25	25	34.83333333	100%	14	25	25
	EXPERIMENT 4					EXPERIMENT 5					EXPERIMENT 6				
Control 1	17.33333333	88%	0	20	22	17.3333333	88%	0	20	22	18.33333333	88%	0	22	22
Control 2	16.5	84%	0	19	21	16.8333333	88%	0	19	22	17.16666667	92%	0	19	23
WWTP A 1	17.83333333	88%	0	21	22	16.3333333	88%	0	18	22	17	84%	0	20	21
WWTP A 2	18.66666667	92%	0	22	23	18.3333333	100%	0	20	25	20	96%	0	24	24
WWTP A 3	18.33333333	88%	0	22	22	19	96%	0	22	24	16.16666667	80%	0	19	20
WWTP B 1	17.16666667	92%	0	19	23	18.8333333	100%	0	21	25	17.66666667	80%	0	22	20
WWTP B 2	15.66666667	80%	0	18	20	16.3333333	88%	0	18	22	20	96%	0	24	24
WWTP B 3	18.33333333	88%	0	22	22	16.1666667	92%	0	17	23	17	84%	0	20	21
WWTP MIXTED 1	17.33333333	88%	0	20	22	17.5	96%	0	19	24	18.33333333	88%	0	22	22
WWTP MIXTED 2	16.16666667	80%	0	19	20	18.3333333	100%	0	20	25	19.16666667	92%	0	23	23
WWTP MIXTED 3	18.66666667	92%	0	22	23	19	96%	0	22	24	20.16666667	92%	0	25	23

Table 4. Letuce Germination in petri dishes. The results correspond to two controls with distillated water and with mixture, black, and gray WWTP sludges. We have the germination for the first, second and third day. Finally, we have the emergency speed index and total percentage of emergence.

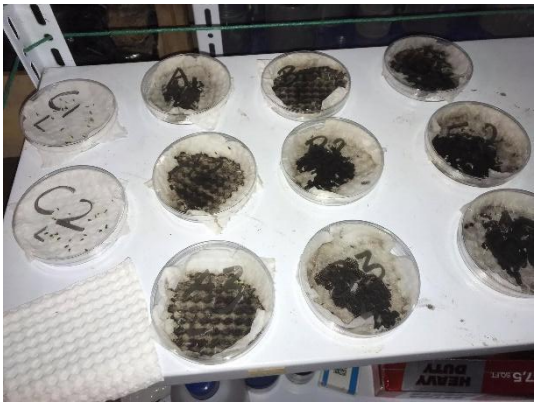

Fig 3. Letucce Germination, third day. These experiments correspond to two controls with distillated water and two with mixture WWTP, two with black WWTP, and two with gray WWTP sludges.

6.3 Germination and development of seedlings in plastic germinators using WWTP sludge

6.3.1 Development of Radish Germination Seedlings in Plastic Germinators

Taking into account that T1 contains the control, where only soil from the pots of the university was placed. T2 is for a dry dilution with 70% regular soil, 20% vermi compost, and 10 % WWTP sludge. T3 was dilution with 70% regular soil, 20% organic compost, and 10 % WWTP sludge. T4 we add dilution with 70% regular soil, 10% vermi compost, 10% organic compost and 10 % WWTP sludge. Finally, T5 one we add dilution with 70% regular soil, 20% vermi compost, and 10 % organic compost.

In Figure 4, we can see that after 15 days, we have that T1 and T4 contain 7 plots with the growth of radish seedlings, while T2 and T3 contain 5, while T5 has 8. On the other hand, at the beginning of the process, at five days, only T3 contained signs of growth, as we can see in table 5 shows us 5 seedlings. On the other hand, T1, T2, T4, and T5 have no growth.

After ten days of sowing, we realized that we had almost all the germinated seedlings we found at the end of the experiment on day 15. T5 shows a more significant number of plots with germination, which may be due to the mixture of two kinds a compost without WWTP sludge.

	REPETITIONS				
Germinated	T1	T2	T3	T4	T5

parcels					
Day 5	0	0	3	0	0
Day 10	6	5	5	7	8
Day 15	7	5	5	7	8

Table 5.Germination of Radish in plastic germinators at 5, 10 and 15 days.

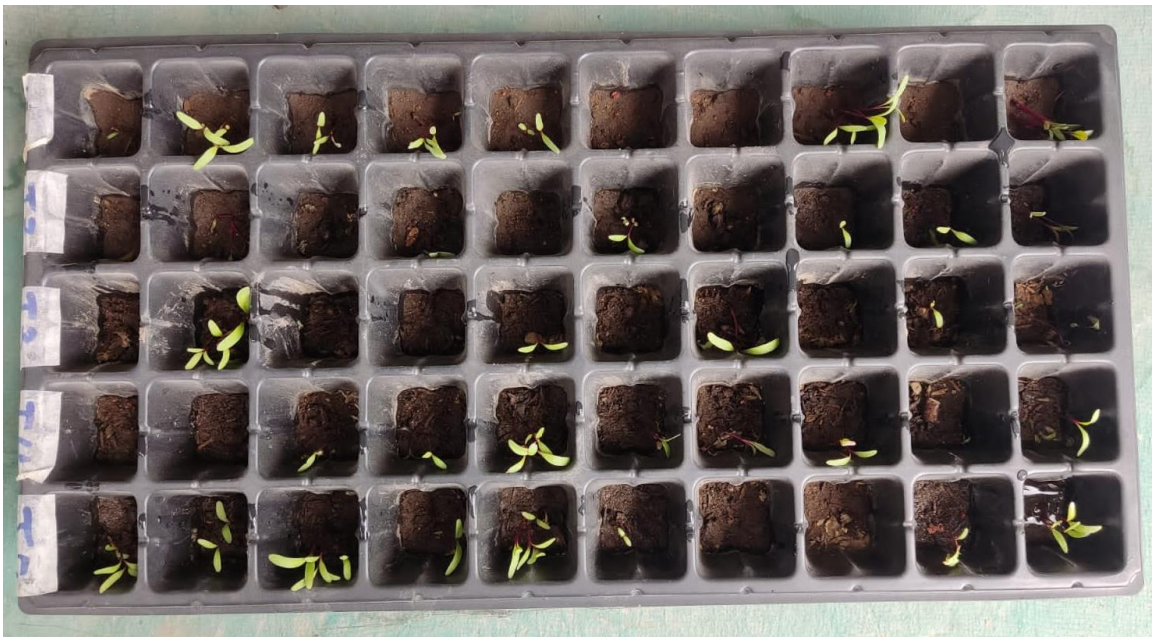

Fig. 4 Germination of Radish in plastic germinators at 15 days

6.3.2 Development of Lettuce Seedlings in Plastic Germinators.

Figure 5 shows a clear difference between T3 and the rest of the plots. We can assume that because T3 include organic compost have a more significant response on the germination. While T1, the control we expected the best response, turns out to be the lowest with a total of 2 germinated plots. Table 5 tells us that we did not have any growth on the first five days, while on day 10, we can already observe a reasonable amount of germination. It can be considered that T2, T3, and T4 had a better response. While T1, with two germinated plots, and T5, with 4 germinated plots, have greater inhibition.

	REPETITIONS

Germinated Parcels	T1	T2	T3	T4	T5
Day 5	0	0	0	0	0
Day 10	1	2	8	4	4
Day 15	2	7	9	8	4

Table 5.Germination of Letucce in plastic germinators at 5, 10 and 15 days.

Fig. 5 Germination of letucce in plastic germinators at 15 days.

6.4 Bacterial identification

Guided by Fig. 1, we can see that in Fig. 6, at 24h, the gray and black water sediments mainly present facultative bacteria. Looking deeper, we realize that we have several bacteria near the surface and others near the bottom, so we can assume that we find anaerobic and aerobic bacteria. At 48 h, we already see a rather cloudy TSB media, so we assume that facultative bacteria continue to predominate. As of 24 hours, a small percentage of anaerobic and aerobic bacteria is still present.

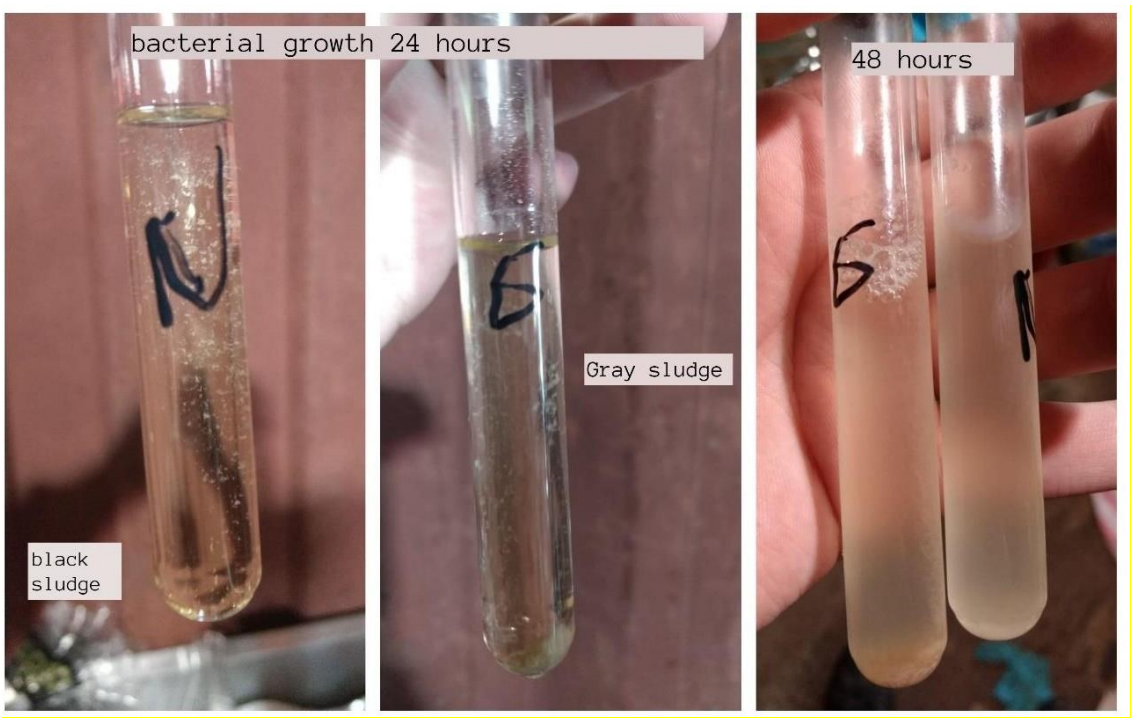

Figure 6: Bacterial growth in test tubes with gray water sludge and black water sludge at 24 and 48 hours. The analysis of these samples was qualitative to observe only the type of bacteria that grew in the tubes.

In Fig.7 we can see a notable difference between the turbidity of the 1:10 (-1) dilution as we carry out the serial dilution until reaching 1:100000 (-5). While the control in Fig. 8 shows us that we do not have contamination in the LB AGAR, nor in the TSB solution before the dilutions.

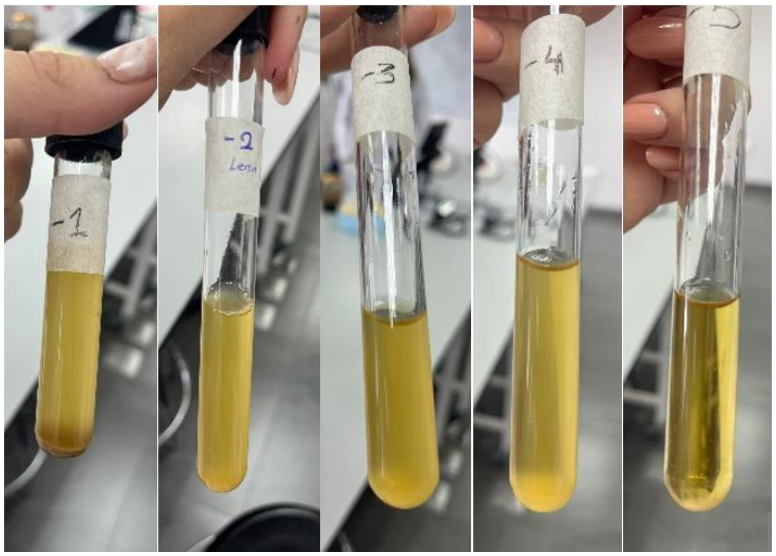

Fig. 7: Bacterial dilutions in test tubes with WWTP sludge with a 1:10 ratio and proceeded to make a serial dilution up to 1:100,000. In order from left to right, -1 has a dilution of 1:10, -2 has 1:100, -3 has 1:1,000,

-4 has 1:10,000, and -5 has 1:100,000.

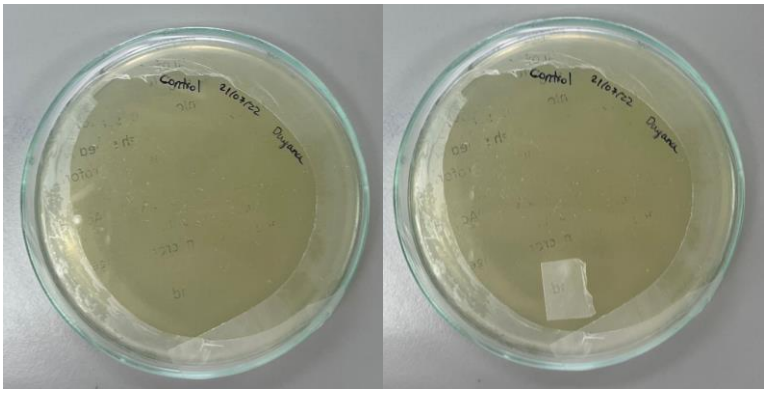

Fig. 8 In this image we find the control, where we test the non-contamination of the agar and the tsb without dilutions.

The bacterial growth that we can observe in Fig. 9 and Table 6 is notably higher in a 1:1000 dilution than in 1:100000. Taking into account that the -3 dilution has a higher bacterial load, it is pretty logical that the difference is so significant, considering values of the -3 solution as 81, 94, 63, 87 and 57 colonies. In comparison, in the -5 dilution, we found only two Petri dishes with bacterial colonies, one with 3 and one with a single colony.

To get the actual values, we must apply the formula Ci x Vi= Cf x Vf, where C is the concentration and V is the volume. With this formula, we can obtain the final concentration of each sample and then count viable bacteria. We need to divide the number of colonies with the ml seeded for this count. After this, we multiply the concentration of our tube with the Dilution Factor.[54]

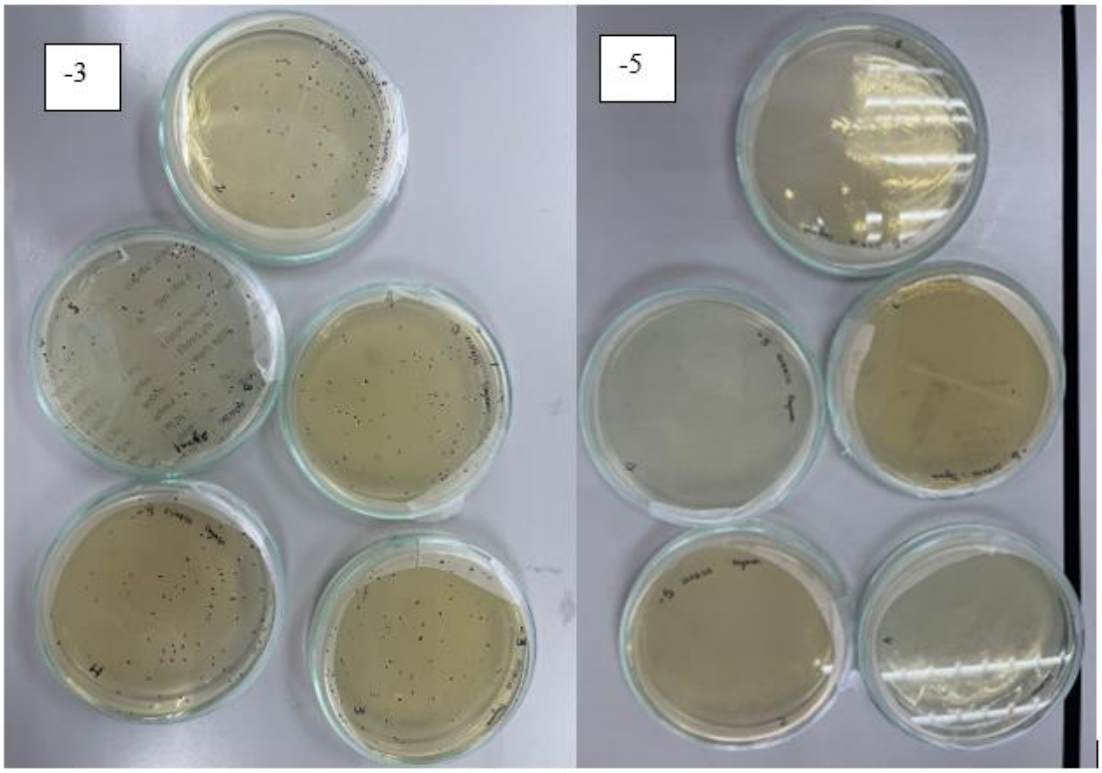

Fig. 9 Bacterial growth in LB AGAR, at 24h with a dilution of 1:1000 (-3), and 1:100000 (-5).

	I	II	III	IV	V
-3 1:1000	81	94	63	87	57
viable bacteria count	810000	940000	630000	870000	570000
-5 1:100000	0	0	3	0	1
viable bacteria count	0	0	3000000	0	1000000

Table 6. Bacterial growth in LB AGAR, at 24h with a dilution of 1:1000 (-3), and 1:100000 (-5).

Tables 6 and 7 shows that we counted a high number of viable bacteria. This would mean a high bacterial load in the WWTP sludge. As we can see, in table 7, after 48h, in the -3 dilution, we reach countless colonies since they exceed 500. However, the load of the -5 dilution can still be counted. The rise in values with a difference of 24h shows us that the bacteria in the WWTP sludge can reproduce exponentially in hours.

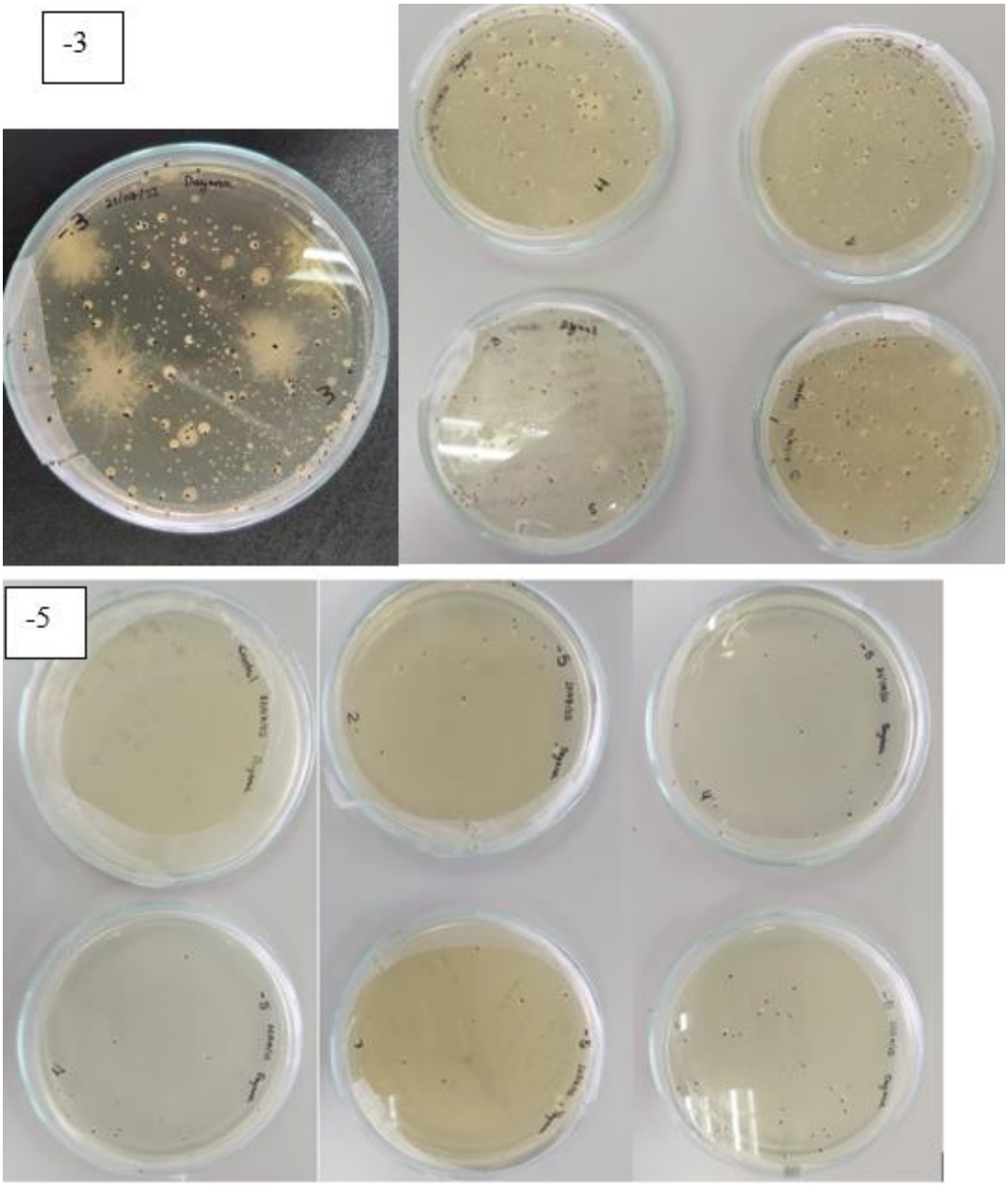

Fig. 10 Bacterial growth in LB AGAR at 48h with a dilution of 1:1000 (-3), and 1:100000 (-5).

	I	II	III	IV	V
-3 1:1000	Uncountable	Uncountable	Uncountable	Uncountable	Uncountable
-5 1:100000	14	17	8	16	35
viable bacteria count (-5)	14000000	17000000	8000000	16000000	35000000

Table 7 Bacterial growth in LB AGAR at 48h with a dilution of 1:1000 (-3), and 1:100000 (-5).

6.5 DNA Extraction

A DNA sample's qualitative and quantitative determination can be vital for the success of subsequent analysis. In most molecular biology laboratories, it is usually carried out using UV-VIS spectroscopy. Analysis of the entire spectral range from 230 to 320 nm is usually recommended for DNA analysis. Within this range, the wavelengths of most significant interest are 260, 280, 230, and 320 nm, respectively.

DNA exhibits maximum absorbance at 260 nm. When measured in a 10 mm cell, the concentration of double-stranded DNA is approximately 50 µg/ml per 1 absorbance unit, using the mean extinction coefficient of 0.020 (µg/ml)-1 cm-1. Therefore, when reading at 260nm, the presence of contaminants, which absorb at 260nm, can cause an overestimation of the DNA content. For this reason, a background correction is usually applied at 320 nm.

The 260/280 absorbance ratio is a good indicator of protein contamination: the DNA sample will be pure if it is less than or equal to 1.8[58,59].

If the 260/230 absorbance ratio is less than 1.8, it indicates the existence of contamination probably caused by organic components or chaotropic agents, which absorb at 230 nm.

In table 8, we can see that our values of 260/280 are around 1.8, where we could find the purity of some of the majority of them. However, in the 260/230 values, we found values much lower than 1.8, which indicates the contamination of the samples [55].

#	Sample ID	Date and Time	Nucleic Acid	Unit	A260 (Abs)	A280 (Abs)	260/280	260/230	Sample Type	Factor
1	WWTP MIXTURE 1	12.08.2022 12:13:43	205,1	ng/µl	4,101	2,274	1,80	1,26	DNA	50,00
2	WWTP MIXTURE 2	12.08.2022 12:15:41	104,5	ng/µl	2,090	1,309	1,60	0,87	DNA	50,00
3	WWTP GRAY 1	12.08.2022 12:17:17	77,6	ng/µl	1,553	1,006	1,54	0,82	DNA	50,00
4	WWTP GRAY 2	12.08.2022 12:18:38	200,0	ng/µl	3,999	2,161	1,85	1,43	DNA	50,00
5	WWTP BLACK 1	12.08.2022 12:20:04	102,4	ng/µl	2,049	1,145	1,79	1,18	DNA	50,00
6	WWTP BLACK 2	12.08.2022 12:21:29	73,3	ng/µl	1,467	0,804	1,82	1,3	DNA	50,00

Table 8. DNA extraction and purification for future sequencing.

6.6 Resistance Genes in Wastewater

The interest of the DNA extraction was to be able to sequence them and thus obtain the resistance genes in the wastewater. Unfortunately, due to lack of time, it will be a job for those who continue in this line of research.

However, advances in genomic research have made it possible to obtain many sequences and identify genes associated with antibiotic resistance. Some databases have been developed that

unify the information. Like, for example, the one developed by the University of Maryland, whose lists include around 20,000 potential ARGs of around 400 types present in bacterial sequences. From these lists, we find the primary genes detected in the influents and effluents of sewage treatment plants, classified according to the family of antibiotics to which they present resistance. This way, we can review Tables 9 through 13. The resistance genes we found in our extracted DNA would be expected to be on this list.[23-25, 56-76]

Gen	Sampling Point				Country	Reference
	Influential	Effluent		Activated Sludge		
bla_{AmpC}	NA	+		+	Germany	Szczepanowski et al., 2009
	+	+	↑	NA	Portugal	Amador et al., 2015
	+	+	↑	NA	Germany	Cacace et al., 2019
	NA	NA		+	China	Yang et al., 2013
bla_{CMY-5}	NA	+		+	Germany	Szczepanowski et al., 2009
bla_{CTX-M}	+	+	↑	NA	Sweden	Bengtsson-Palme & Larsson, 2016
	+	+	↓	NA	Canadá	Bourgin et al., 2018
	NA	+		NA	Spain	Marti et al., 2013
	NA	+		NA	Poland	Korzeniewska et al., 2013
bla_{OXA}	+	+	↓	+	China	Yuan et al., 2014
	+	+	↑	NA	Portugal	Amador et al. 2015
bla_{OX2-1}	NA	+		+	Germany	Szczepanowski et al., 2009
	NA	+		NA	Poland	Korzeniewska et al., 2013
bla_{OXA-5}	NA	+		+	Germany	Szczepanowski et al., 2009
bla_{OXA-48}	NA	+		+	Germany	Szczepanowski et al., 2009
	NA	NA		+	Sweden	Bengtsson-Palme & Larsson, 2016
bla_{OXA-56}	NA	+		+	Germany	Szczepanowski et al., 2009
	+	+	↓	NA	Denmark	Laht et al., 2014
	+	+	↓	NA	Finland	Hultman et al., 2018
bla_{SHV}	NA	+		NA	Spain	Chi et al., 2013
	NA	+		NA	Ireland	Galvin et al., 2010
bla_{TEM}	+	+	↑	NA	Canadá	Biswal et al., 2014
	+	+	NA	+	China	Jiao et al.. 2018
	NA	+		NA	Spain	Chi et al., 2013
	NA	+		NA	Ireland	Galvin et al., 2010
	+	+	↑	NA	Portugal	Amador et al., 2015
	+	+	↑	NA	Canadá	Durrer et al., 2017
	+	+	↑	NA	Spain	Rodriguez-Mozaz et al., 2015
bla_{TEM-1}	NA	+		NA	Poland	Korzeniewska et al., 2013
	+	+	NA	+	Poland	Korzeniewska et al., 2013
	NA	+		NA	Spain	Marti et al., 2014
	NA	NA		+	China	Yang et al., 2013

Table 9. Resistance genes to beta-lactam antibiotics detected in WWTPs.

Gen	Sampling Point				Country	Reference
	Influential	Effluent		Activated Sludge		
ermA	NA	-		+	Germany	Szczepanowski et al., 2009
ermB	NA	+		+	Germany	Szczepanowski et al., 2009
	NA	NA		+	China	Yang et al., 2013
	+	+	↓	+	China	Yuan et al., 2014
	+	+	NA	+	China	Jiao et al., 2018
	NA	+		NA	Spain	Marti, Variatza, et al., 2014
	NA	+		NA	Netherlands	Sabri et al., 2020
	+	+	↓	NA	Germany	Alexander et al., 2015
	+	+	↓	NA	Canadá	Durrer et al., 2017
	-	+		NA	USA	Bergeron et al., 2015
	+	+	↑	NA	Spain	Rodriguez-Mozaz et al., 2015
ermF	NA	+		+	Germany	Szczepanowski et al., 2009
	NA	NA		+	China	Yang et al., 2013b
	+	+	↓	+	China	Yuan et al., 2014

Table 10. Resistance genes to macrolide antibiotics detected in WWTPs.

Gen	Sampling Point				Country	Reference
	Influential	Effluent		Activated Sludge		
qnrA3	NA	+		+	Germany	Szczepanowski et al., 2009
qnrB1	NA	+		+	Germany	Szczepanowski et al., 2009
qnrS	NA	+		NA	Spain	Marti et al., 2013
	NA	+		NA	Spain	Marti, Huerta, et al., 2014
	-	-		+	China	Xu et al., 2015
	+	+	↑	NA	Canadá	Durrer et al., 2017
	+	+	↑	NA	Spain	Rodriguez-Mozaz et al., 2015

Table 11. Quinolone antibiotic resistance genes detected in WWTPs.

Gen	Sampling Point				Country	Reference
	Influential	Effluent		Activated Sludge		
dfrII	NA	+		+	Germany	Szczepanowski et al., 2009
sulI	NA	+		+	Germany	Szczepanowski et al., 2009
	NA	NA		+	Poland	Ziembinska-Buczynska et al., 2015
	NA	NA		+	China	Yang et al., 2013b
	NA	NA		+	China	D. D. Zhang et al., 2011
	NA	NA		+	China	Y. Zhang et al., 2016
	NA	+		NA	Spain	Marti et al., 2013
	NA	+		NA	Netherlands	Sabri et al., 2018
	+	+	↓	NA	Denmark	Laht et al., 2014
	+	+	↓	NA	Canadá	Durrer et al., 2017
	+	+	↑	NA	Spain	Y. Zhang et al., 2016
	+	+	↑	NA	Canadá	Marti, Huerta, et al., 2014
	+	+	↓	+	China	Xu et al., 2015
	+	+	↑	+	China	Yuan et al., 2014
	+	-	↓	NA	USA	Bergeron et al., 2015
sulII	NA	+		+	Germany	Szczepanowski et al., 2009
	NA	NA		+	Poland	Ziembinska-Buczyrska et al., 2015
	NA	NA		+	China	D. D. Zhang et al., 2011
	NA	NA		+	China	Yang et al., 2013b
	NA	NA		+	China	Y. Zhang et al., 2016
	+	+	↓	+	China	Yuan et al., 2014
	+	+	↑	+	China	Xu et al., 2015
	+	+	NA	+	China	Jiao et al., 2018
	+	+	↑	NA	Canadá	Biswal et al., 2014
	+	+	↓	NA	Denmark	Laht et al., 2014
	NA	+		NA	Spain	Marti et al., 2013
	NA	+		NA	Netherlands	Sabri et al., 2018
	+	+	↑	NA	Canadá	Durrer et al., 2017
sulIII	NA	+		+	Germany	Szczepanowski et al., 2009
	NA	NA		-	Poland	Ziembinska-Buczynska et al., 2015
	NA	NA		+	Sweden	Bengtsson-Palme & Larsson, 2016
	-	+	↑	NA	Canadá	Biswal et al., 2014
	+	+	↓	+	China	Xu et al., 2015

Table 12. Resistance genes to sulfonamide and trimetropim antibiotics detected in WWTPs.

Gen	Sampling Point				Country	Reference
	Influential	Effluent		Activated Sludge		
tetA	NA	+		+	Germany	Szczepanowski et al., 2009
	+	+	↑	NA	Canadá	Biswal et al., 2014
	NA	+		NA	Poland	Harnisz et al., 2015
	NA	NA		+	China	Yang et al., 20136
	NA	NA		+	China	X.-X. Z. and T. Zhang, 2011
	NA	NA		`	China	D. D. Zhang et al., 2011
	+	+	↓	+	China	Yuan et al., 2014
	+	+	↓	+	China	Xu et al., 2015
	+	+	↓	+	China	Pei et al., 2019
	-	-		NA	USA	Bergeron et al., 2015
tetB	NA	+		NA	Poland	Harnisz et al., 2015
	NA	NA		+	China	X.-X. Z. and T. Zhang, 2011
	+	+	↓	NA	Canadá	Biswal et al., 2014
	+	+	↓	+	China	Xu et al., 2015
tetE	+	+	↑	+	China	Xu et al., 2015
	+	+	NA	+	China	Jiao et al., 2018
	-	-		NA	Canadá	Biswal et al., 2014
	NA	NA		+	China	Zhang, 2009
	NA	NA		+	China	X.-X. Z. and T. Zhang, 2011
tetH	NA	+		+	Germany	Szczepanowski et al., 2009
	-	-		NA	Canadá	Biswal et al., 2014
tetR(31)	NA	+		+	Germany	Szczepanowski et al., 2009
tetW	NA	NA		+	China	Yang et al., 2013b
	NA	NA		+	China	D. D. Zhang et al., 2011
	+	+	↓	+	China	Yuan et al., 2014
	+	+	↓	+	China	Xu et al., 2015
	+	+	↑	NA	Spain	Rodriguez-Mozaz et al., 2015
	NA	+		NA	Spain	Marti et al., 2013
	NA	+		NA	Netherlands	Sabri et al., 2018
	-	-		NA	USA	Bergeron et al., 2015

Table 13. Tetracycline antibiotic resistance genes detected in WWTPs.

6. DISCUSSION

Hazardous waste, endowed with corrosive, reactive, explosive, toxic, flammable, and biological-infectious properties (CRETIB characteristics), have been subject to environmental regulation for several years in various parts of the world.

The environmental implications of the CRETIBs could lead to innumerable problems.

The pH and dissolved oxygen obtained from the WWTP measurements indicate good plant management. An almost neutral pH was achieved, which is the expected value. On the other hand, we have dissolved oxygen which shows us an excellent percentage. The pH and dissolved oxygen values are ideal for marine life or human consumption. However, the treated wastewater is not considered potable, so it is used for irrigation.

Knowing that the wastewater is for irrigation, a germination experiment was carried out, where we can calculate the emergency speed index and the percentage of total emergence. These values indicate the seed growth speed and the hatched seeds' percentage. The values found are encouraging. It was found that the seeds of both radish and lettuce did not present any inhibition against the dilutions with WWTP. There are even values where they respond better with sludge than without. This may be because the controls only have distilled water. From this, we could assume that not only do they not present a risk, but they also provide nutrients for the growth of the different spices. However, we must be sure of this with a laboratory study of the sludge content.

Adding further study to the germination of plants, the study of germination in plastic germinators was also carried out. In this, not only a minimum amount of sludge is handled, but also a considerable amount to notice the changes on a larger scale. In this experiment, we also used two types of compost created by the SOL GOLD company. These composts are created from daily kitchen waste. For its part, Vermicompost is a treatment of larvae given to organic waste. On the other hand, organic compost is created from the controlled putrefaction of organic waste.

Germination and growth of radish seeds were relatively similar. On day 15, we had the most growth in the plot, including the soil from the university gardens, together with the two composts. At the same time, those that presented less amount of germination were those that contained sewage sludge. However, they do not follow a pattern since the germination of lettuce is different. In the germination of lettuce, we found more excellent germination in the plot where there was regular soil, organic compost, and WWTP sludge. The least growth is found in the first plot, which gives us only two germinated plots. With these results, we can assume that the sewage sludge has

a better result or behavior when mixed with the compost. Furthermore, in the same way, it would not present a higher risk rate in the environment.

On the other hand, with a somewhat disconsolate response, we find bacterial growth. In the TSB experiments at 24h and 48h, we have many facultative bacteria that can represent a significant risk for the environment and the proliferation of bacteria. Therefore, it was necessary to carry out serial dilutions to obtain a count of bacteria in the Petri dishes with LB AGAR. With a dilution of 1:100,000 at 48h, we achieved an alarming bacterial growth. This indicates that the bacteria found in the WWTP sludge have a rapid and easy proliferation, which must be considered.

In the results of the DNA extraction, we found many impurities that did not allow sequencing to identify the resistance genes. This procedure would need to be improved in order to achieve greater precision. In the case of finding resistance genes mentioned in "Resistance Genes in Wastewater" results, we must find agents that control this problem that is getting bigger every day.

7. CONCLUSION AND RECOMMENDATIONS

The typical characteristics of raw wastewater are, as we saw in Table 1, full of minerals and microorganisms that are expected to be counteracted as much as possible for future use. For this reason, WWTPs must be monitored to avoid releasing inorganic substances, such as minerals and metals that are not biodegradable.

Although the results showed us a pretty good pH and dissolved oxygen, there are other factors that influence the purity of the water. Therefore, these values must continue to be maintained in order to discard them in future studies and to be able to focus on the other problems that revolve around this topic. This point in favor tells us that the wastewater we are studying does not represent a corrosive, reactive or explosive risk.

Germination experiments show that wastewater does not threaten the average growth of generic plants such as radishes and lettuce. However, if we want a better result, we must put low amounts of mud and high amounts of fertile soil and compost. Considering that many endemic species tend to be much more delicate than the ones we experience, it is recommended to only expel this sludge into the environment after first being treated and mixed. Furthermore, even, it should be considered to carry out additional studies of the damages or benefits that sewage sludge has on the species that could be found in the area of interest.

On the other hand, the organic substances found in wastewater are commonly used as food for microorganisms. Even so, many synthetic organic compounds found in the WWTP are not biodegradable and increase the sedimentation rate of water bodies. This can be verified in the bacterial growth in the Petri dishes. Although there are high dilutions, it can be seen that from 24h to 48h, there is a rapid increase in colonies. This problem is found within the CRETIB, where they represent a biological and environmental problem. The rapid proliferation of bacteria and viruses can lead to several diseases, not only in humans. Many flora and fauna could be affected by this problem.

Some biological treatments can be applied to wastewater to achieve greater purity and less contamination. Indeed, it is recommended to treat these problems before they are released into the environment. In this way, we can prevent many environmental problems.

Finally, as we mentioned in the THEROICAL BACKGROUND, the use and overuse of antibiotics have had a severe environmental impact. Knowing also that one of the main entrances to the environment of these drugs is wastewater since between 40 and 90% of antibiotics are not metabolized through biochemical pathways and are excreted through urine or feces, both in humans and in animals arriving at the treated wastewater stations. Strategies must be created to eliminate them so they do not generate a worse catastrophe.[78]

In Colombia, to optimize the effectiveness of WWTPs, engineer Agudelo, under the direction of Professor Cardona in his doctoral research, developed a kinetic model to mathematically simulate how meropenem would degrade with the proposed catalytic ozonation system.[77]

The first stage of his work involved resolving the problem that hospital wastewater, like the ones to be treated, is not separated. That is why he created a sequential treatment system using vermifiltration, for which there is a physical medium of worms and filter materials such as sand of different sizes. This was used to separate the coarse organic matter and a large part of the dissolved one present in the liquid, which, although it was not an objective of the study, allowed it to reduce a part of the meropenem. [77]

To eliminate the remaining residues of the antibiotic, the water was treated with hydroxyl radicals. These molecules would be in charge of oxidizing or reducing the drug and degrading it, with the advantage that they are harmless to the environment and have a more significant potential to clean wastewater, even more than chlorine.[79, 56] To achieve these oxidants, two low-cost and sustainable technologies were used. One of them was using Portland cement (in paste) to increase the pH or

acidity of the water, and the other was powdered activated carbon together with ozone. [77]
Although this was a doctoral thesis, which became a pilot plan, as we can see, they have very positive results that can be replicated and industrialized in all types of wastewaters.

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