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Spatial Study of Enzymatic Activities from Bacterial Isolates in a Mediterranean Urban Park

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Abstract: Urban parks are a rich source of microbial diversity, as they are heavily used by city residents. In this study, we sampled a Mediterranean park and were able to isolate bacteria that have the ability to inhibit the growth of control microorganisms. Out of the 560 bacteria we tested, many displayed antibacterial activity, particularly against *Salmonella* sp. and *K. pneumoniae*. These results suggest that the microorganisms in the park are in close proximity to the human population. Additionally, the isolated bacteria demonstrated diverse enzymatic activities, possibly as a response to the environmental substances available to them, which could aid in the degradation of different compounds of interest. The study of the spatial distribution of soil parameters and the inhibition against relative-safe pathogens in an urban park in València (Spain) demonstrated a higher proportion of isolates in certain areas. These spatial data maps can help researchers understand the behaviors of bacterial populations on a regional level, which can assist in the creation of novel antimicrobial agents and promote advancements in public health.

Keywords: urban garden; protease; lipase; DNase; lipase; spatial statistics

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1. Introduction

Urban gardens play a role in maintaining biodiversity in anthropogenic areas. Their potential value may be considerable, as they can function as semi-natural habitats that offer a sanctuary for bacterial biodiversity [1]. Some of these bacteria include strains that are able to produce antibiotics and other interesting products for the industry, such as extracellular enzymes [2]. Additionally, anthropogenic activities frequently impact urban garden soils, potentially leading to bacterial community composition variations distinct from those observed in conventional agriculture. Such variations can manifest in antibiotic-producing soil strains [2]. Urban gardens often constitute a significant proportion of green spaces in urbanized cities, with some cities having 23 to 36% of their areas dedicated to these gardens [3–5]. These gardens provide support for numerous local, landscape, and sociopolitical features that aid in the preservation of biodiversity. By comprehending spatial connectivity, it is possible to anticipate bacterial resource diversity, abundance, distribution, composition, and species distribution. Opportunistic pathogens that are typically present in soil microbiota or those that colonize it (enteric pathogens) can contaminate the soil through the deposition of human or animal excreta. Animal-based excreta, such as manure, and the improper disposal of human excrement in gardens can introduce substantial quantities of enteric pathogens into the soil environment. The emergence of antimicrobial resistance in urban areas should not be ruled out either [6]. One of the major global health concerns is antibiotic-resistant bacteria with ubiquitous phenotypes and genotypes in parks and gardens [7]. Matthiessen et al. [8] advocate that the high prevalence of antibiotic-resistant bacteria in the environment is one of the most important threats to public health today due to their direct contact with humans. The overuse and misuse of antibiotics

have inevitably increased the environmental concentration of antibiotic resistance among bacteria, especially among enterobacteria [9,10]. The problem of antimicrobial resistance in parks is only becoming worse [11]. John Snow's work on cholera and the acceptance of germ theory show that urban dwellers interact with microbes in markedly different ways than those living in rural areas [12]. Microbes in an urban environment are a potential source of contagion [13], and certain conditions, such as allergies, are associated with increasing urbanization [14]. It is more than evident that cities (and, thus, their parks and gardens) have an impact on human health. The mechanisms of this impact are variable and not well understood. The analysis and mapping of microbial dynamics in these urban environments outside pandemics have only just begun [15]. The use of maps for analyzing and illustrating the occurrence of bacterial pathogens at a national level has been employed on a European-wide basis since 1999 [16]. Their contribution to predicting urban environments will enable significant new research on the impact of urban microbiomes on human health [17].

The present investigation scrutinized bacterial resources within an urban garden situated in València (Eastern Spain) to establish the relationship between alterations in soil composition and bacterial abundance, as well as the spatial distribution of bacteria within the garden. Benicalap park is one of the most important green spaces in the city of València. It is an outdoor space that has large sports areas, swimming pools, a theater forum, and children's areas. The park was built in 1983 on the periphery of the city. It covers an approximate area of 80,000 m^2. Vegetation is present in most of the park in the form of Mediterranean wooded groves, e.g., olive trees, cypresses, pines, laurels, holm oaks, mulberry trees, and strawberry trees. The park can be accessed via Burjassot Avenue, Luis Braille Street, and Andreu Alfaro Street. In this study, the microbiological biodiversity was specifically tested, e.g., fungi and bacteria (Enterobacteria). In total, 28 samples were studied extensively in terms of soil bacteria composition, antibiotic effects against test strains, and the ability to produce extracellular hydrolytic enzymes. Bacteria and fungi are incapable of performing endocytosis processes, which prevents them from ingesting nourishing particles or taking advantage of macromolecules in suspension in the external environment as sources of nutrients. It is not uncommon for these microorganisms to produce and excrete medium enzymes with the ability to extracellularly hydrolyze various types of macromolecules, producing smaller molecules (monomers, dimers, oligomers) that will later be incorporated by active transport to the cytoplasm. Moreover, we examined the relationship between bacterial abundance, spatial distribution, and other soil characteristics, such as pH and color.

2. Materials and Methods

2.1. Sample Collection

During the 2018 and 2019 seasons, soil sampling was carried out in an urban community park located in Benicalap, València, Spain (Figure 1). The garden utilized municipal and rainwater for irrigation, which is a common practice in many urban community gardens throughout València. To isolate bacteria, a total of 28 soil samples were collected from a depth of 0–10 cm and transferred to screw tubes, following the procedures detailed in a prior publication [18]. The unused samples were kept at 4 ºC for subsequent analysis.

Figure 1. Park in Benicalap. Geolocation of sampling points (Google Maps).

2.2. Soil Characterization

2.2.1. pH Measurement

To determine soil pH, 1 g of soil was dissolved in 5 mL of distilled water, agitated for 2 min, and allowed to settle for 30 min. The soil pH was determined in triplicate using a pH meter (Consort, Turnhout, Belgium) (Figure 2).

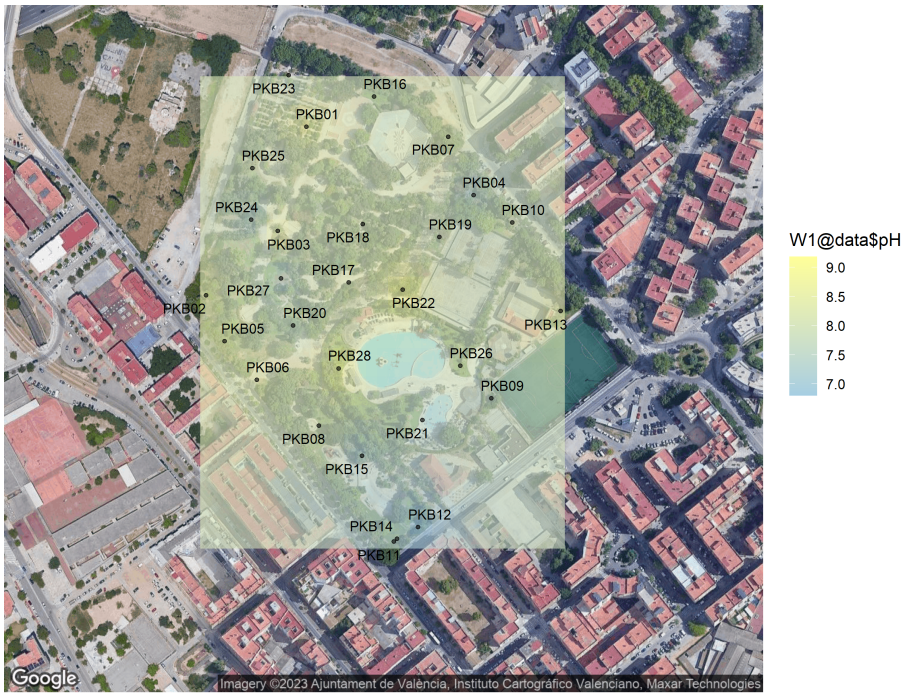

Figure 2. Kriging distribution of pH measurements (black dots) at Benicalap park.

2.2.2. Color and Texture Determination

The soil color was assessed through a visual examination of the samples and compared against a Munsell standard table [19]. The texture of the soil was determined to enable classification based on the sizes of the constituent particles, as described elsewhere [20–22].

2.3. Isolation and Antibiosis Characterization of Soil Bacteria

To prepare serial dilutions, 1 g of the sample was suspended in 10 mL of sterile water, and 0.1 mL of the resulting 1:10 to 1:100,000 dilutions were plated on 10% trypticase soy agar (TSA, Conda, Spain). After an incubation period of 48 h, 15–20 random colonies were chosen and transferred onto a fresh TSA "mother" plate using a grid. Calibrated suspensions of safe relatives (*Escherichia coli* CECT101, *Bacillus cereus* CECT495, *Salmonella sp.* CECT443, *Staphylococcus aureus* CECT4013, *Pseudomonas fluorescens* CECT378, *Klebsiella pneumoniae* CECT143, *Enterobacter cloacae* CECT194, or *Enterococcus faecalis* CECT184) were spread on TSA plates using sterile swabs. All grown microorganisms from the mother grid plate were individually replicated on these plates using sterile toothpicks to evaluate their antibiotic effects.

2.4. Microbial Identification

2.4.1. 16S rDNA Partial Sequence

Bacterial DNA was extracted and amplified following the method described by Arahal et al. [23]. The 16S rRNA gene was amplified using primers SWI-F (5′-AGAGTTTGATCCTG GCTCAG-3′) and SWI-R (5′-GGTTACCTTGTTACGACTT-3′) [24]. The amplification reaction was performed in a Primus 25 thermocycler (MWG, Ebersberg, Germany). Amplification products were separated by electrophoresis on a 1% (*w*/*v*) agarose gel. The PCR amplifications were purified and washed using a high-pure PCR product amplification kit (Boehringer, Mannheim, Germany). The direct sequencing of the PCR products was carried out using the ABIPrism BigDye Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems, Stafford, TX, USA) at the SCSIE service (Universitat de València, Paterna, Spain). The sequences were aligned using the BLAST program with complete 16S rDNA gene sequences obtained from the EMBL nucleotide sequence data libraries [25].

2.4.2. MALDI-TOF

The bacterial strain identification was conducted following the recommended protocol by Bruker Daltonics (http://www.bdal.de) utilizing the extended direct transfer method. Freshly cultured strains were analyzed using the matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) technique. A Microflex L20 mass spectrometer (Bruker Daltonics) equipped with an N2 laser was utilized, and all spectra were acquired in a positive linear ion mode with an acceleration voltage of 20 kV [26]. The spectra were acquired as the sum of 240 shots per target within the mass range of 2000–20,000 Da. Three spectra were obtained per strain using the MALDI Biotyper Real-time Classification (RTC) method, and the resulting identification against the database MBT 7854 and MBT 7311_RUO (Bruker Daltonics) corresponded to the profile with the highest log score.

2.5. Assays for Extracellular Hydrolytic Enzymes Production

To demonstrate the production of extracellular enzymes (hydrolases), the microorganisms were grown in a standard culture media, provided with the basic nutrients necessary for good growth of the strain, and supplemented with the macromolecule under investigation (proteins, polysaccharides, lipids, or nucleic acids). After timely incubation, the presence of the macromolecule was investigated using an appropriate reagent to show its presence. Sometimes the presence of the macromolecule gives the environment a characteristic aspect (opacity, solidity). This disappearance will indicate that hydrolysis has occurred.

All of the strains isolated in this project were spread onto four replicated Petri plates using sterile toothpicks, each containing media with the following composition in g L^{-1}.

- Casein agar: 10 bacteriological peptone, 4 NaCl, 3 meat extract, 15 agar; 10% of skim milk was sterilized in a separate vessel. Once sterile, both parts were poured onto Petri plates. The plates were incubated at 28 ºC for 3 days. The presence of casein gives the environment an opaque appearance that disappears when casein is hydrolyzed. A transparent halo must appear in the middle of the proteolytic microorganisms grown on casein. The non-proteolytic ones do not produce a change in the original aspect of the environment.
- TWEEN-80 agar: This medium consists of a synthetic lipid containing ester links between sorbitol and oleic acid (Tween-80) and calcium salts. When microorganisms possessing esterase activity (lipase) are present, they can hydrolyze the ester link, leading to the release of oleic acid from Tween-80. In the presence of an excess amount of Ca^{2+}, the released oleic acid forms small crystalline oleate crystals that create an opaque halo around the growth area.
- Starch Agar: To reveal the presence of starch, it was necessary to dye it with Lugol. We added 2 milliliters of Lugol to the plate and observed the development of the dark violet color in areas where there was starch. If transparent halos appeared around the growth of a microorganism, this indicated that there was polysaccharide hydrolysis.
- DNase agar: 20 tryptose, 2 deoxyribonucleic acid, 5 sodium chloride, 12 agar. The agar medium was inoculated and incubated with the organisms, then the surface growth was flooded with 1N hydrochloric acid. Polymerized DNA, which is present in the medium, will precipitate in the presence of 1N HCl and cause the medium to become opaque. If the organisms produce enough DNase enzymes to hydrolyze the DNA, clear zones will be observed around the colonies. As a control, *Staphylococcus aureus* CECT4013 was used.

2.6. Kriging Method for Biological Spatial Statistics

Spatial statistics is a collection of methods used to analyze and interpret geographical data by using various interpolation techniques such as inverse distance weighted (IDW), splines, and Kriging. IDW is a simple technique that predicts outcomes based on nearby sample values, but it can produce a bull's-eye pattern and an uneven surface. Splines also create a smooth surface by fitting a mathematical function to the input data, but they are deterministic methods. Kriging, on the other hand, is a statistical method that incorporates spatial autocorrelation and is preferred for spatial interpolation [27].

The soil samples yield valuable information that is recorded as a dataset linking specific locations to the GPS coordinates, longitude, and latitude. The data are represented by the number of colonies or isolates that inhibit the growth of control strains at a given location x, denoted as $Y(x)$. The dataset is defined as $Y(x),, x \in \mathcal{D}$, where $\mathcal{D}$ includes all of the locations being analyzed. Kriging techniques [28] are then utilized on this dataset to facilitate spatial analysis.

3. Results and Discussion

Urban park soil is capable of sustaining microbial life in different degrees and conditions. Depending on the degree of anthropogenization, areas with higher concentrations of microorganisms are observed (top-right and bottom-left extremes) Figure 3.

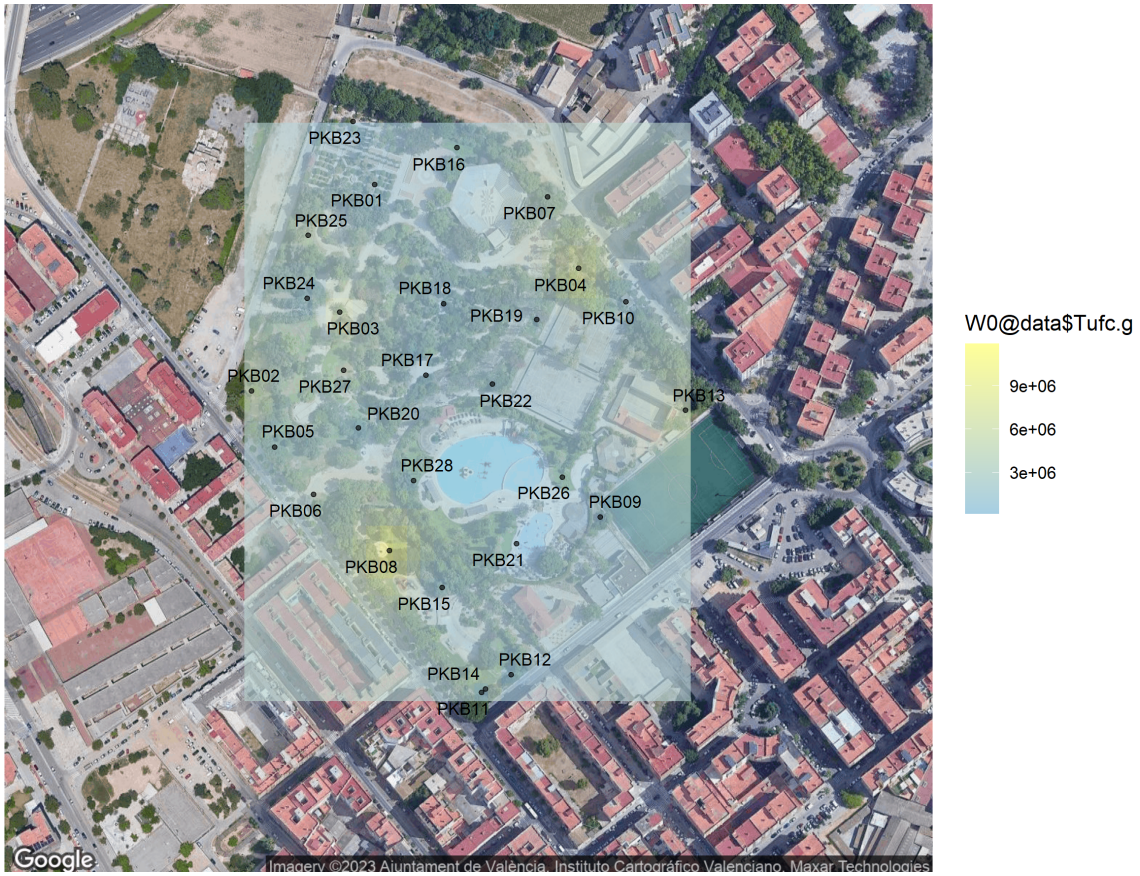

Figure 3. Kriging mapping of isolated microorganisms at the park in Benicalap.

Based on the possible different characteristics of these soils, different tests were carried out and they allowed us to characterize them minimally. On the one hand, one of the most significant properties of the soil, the color, was determined. It was generally conditioned by the existence and proportion of organic and mineral compounds.

Organic matter produces dark colors, usually blackish or brown. Most of the world's soils are dominated by two large lineages of bacteria, i.e., *Proteobacteria* and *Actinobacteria* [29]. They are very efficient competitors for organic carbon, which is a limiting resource in the soil. Therefore, their abundance increases the concentrations of organic carbon. The darker the soil, the greater the chance of finding a large number of microorganisms. Most of the analyzed soils showed a dark or brownish-dark coloration, which suggests a great number of organic compounds and, consequently, high numbers of microorganisms (Table 1).

Although the samples were taken at different depths, ranging from the surface to 10 cm, similar results were observed. The soil type is strongly associated with pH, which is determined by the constituents of the soil. The pH value influences the type of microorganisms that can be found in each soil sample. Bacteria prefer neutral or slightly alkaline conditions, while fungi are more abundant at acidic pH [29]. In the soils in Mediterranean regions, the calcium content is high and the pH is alkaline [30]. The pH values in these soils are linked to the presence or absence of $CaCO_3$ [31].

The soils analyzed exhibited a range of pH from 6.79 to 9.24. We decided to only isolate bacteria for further experiments, following previous strategies [32,33].

Table 1. Geo-location and basic soil characterization.

Sample Code	GPS Coord	Depth	pH	Color	Texture	Total ufc·g^{-1}
PKB01	39.4986, −0.3973	2–5	8.71	Dark	Sandy-loam	1.70×10^5
PKB02	39.4974, −0.3982	0–2	8.46	Brown	Sandy-loam	4.70×10^6
PKB03	39.4979, −0.3976	0–2	8.63	Brownish dark	Sandy-loam	6.00×10^6
PKB04	39.4981, −0.3958	2–5	7.60	Grey	Sandy-loam	9.00×10^6
PKB05	39.4971, −0.3981	0–2	8.57	Brown	Sandy-loam	9.40×10^5
PKB06	39.4968, −0.3978	0–2	8.53	Dark	Sandy-loam	3.20×10^6
PKB07	39.4985, −0.3960	5–10	8.48	Dark	Sandy-loam	4.00×10^6
PKB08	39.4965, −0.3972	2–5	8.19	Dark	Sandy-loam	1.20×10^7
PKB09	39.4967, −0.3957	2–5	7.80	Dark	Sandy-loam	7.40×10^5
PKB10	39.4979, −0.3955	0–2	8.13	Brown	Sandy-loam	7.00×10^5
PKB11	39.4957, −0.3965	2–5	7.50	Dark	Sandy-loam	6.60×10^5
PKB12	39.4958, −0.3963	2–5	6.79	Brownish dark	Sandy-loam	9.70×10^4
PKB13	39.4973, −0.3950	2–5	8.68	Brownish dark	Sandy-loam	6.40×10^6
PKB14	39.4957, −0.3965	0–2	7.21	Brownish dark	Sandy-loam	5.70×10^6
PKB15	39.4963, −0.3968	0–2	7.33	Brown	Sandy	6.60×10^5
PKB16	39.4988, −0.3967	2–5	8.00	Dark	Sandy	4.60×10^5
PKB17	39.4975, −0.3969	0–2	8.33	Dark	Sandy-loam	3.00×10^5
PKB18	39.4979, −0.3968	0–2	8.28	Bright	Sandy	4.90×10^5
PKB19	39.4978, −0.3961	2–5	8.09	Dark	Sandy-loam	1.20×10^6
PKB20	39.4972, −0.3974	0–2	7.89	Dull	Sandy	5.60×10^5
PKB21	39.4966, −0.3963	0–2	7.63	Light	Sandy	1.20×10^5
PKB22	39.4975, −0.3965	5–10	9.24	Dull	Sandy-loam	9.30×10^5
PKB23	39.4990, −0.3975	2–5	7.85	Dark	Sandy	4.30×10^4
PKB24	39.4980, −0.3978	0–2	7.55	Brownish dark	Sandy-loam	6.80×10^5
PKB25	39.4983, −0.3978	2–5	8.11	Brownish dark	Sandy-loam	4.10×10^6
PKB26	39.4969, −0.3959	2–5	8.06	Dull orange	Sandy	7.20×10^5
PKB27	39.4976, −0.3976	2–5	7.47	Grey	Sandy-loam	3.30×10^6
PKB28	39.4969, −0.3970	0–2	9.00	Dull orange	Sandy-loam	7.30×10^5

Microorganisms living in soil are in constant competition with other microbiota that share their niche. To evaluate the ability of soil bacteria to inhibit the growth of other bacteria, inhibition tests were performed [33]. After a 24-hour incubation period, calibrated suspensions of safe bacterial strains, including *E. coli*, *S. aureus*, *K. pneumoniae*, *Salmonella* sp., *P. fluorescens*, and *Ent. cloacae*, were spread on TSA plates using sterile swabs. The grown microorganisms from the mother grid plate were then individually replicated on these plates using sterile toothpicks to check for antibiotic effects (Table 2). A Kriging representation of these results is shown in Figure 4.

Among the 560 bacteria analyzed, a variable amount of antibiosis was detected, with *Salmonella* sp. and *K. pneumoniae* having the highest number of microorganisms with antibiotic effects. On the other hand, antibiosis against *P. fluorescens* and *Ent. cloacae* were almost anecdotal.

However, urban parks are home to a large number of microorganisms that can be sources of enzymes with industrial applications. A set of four biochemical tests was performed to detect possible enzyme producers of interest with DNase, lipase, amylase, and protease activities among all isolated bacteria (Table 3). Between 10 and 24 isolates tested positive. For the distribution of the results (Kriging representation), see Figure 5.

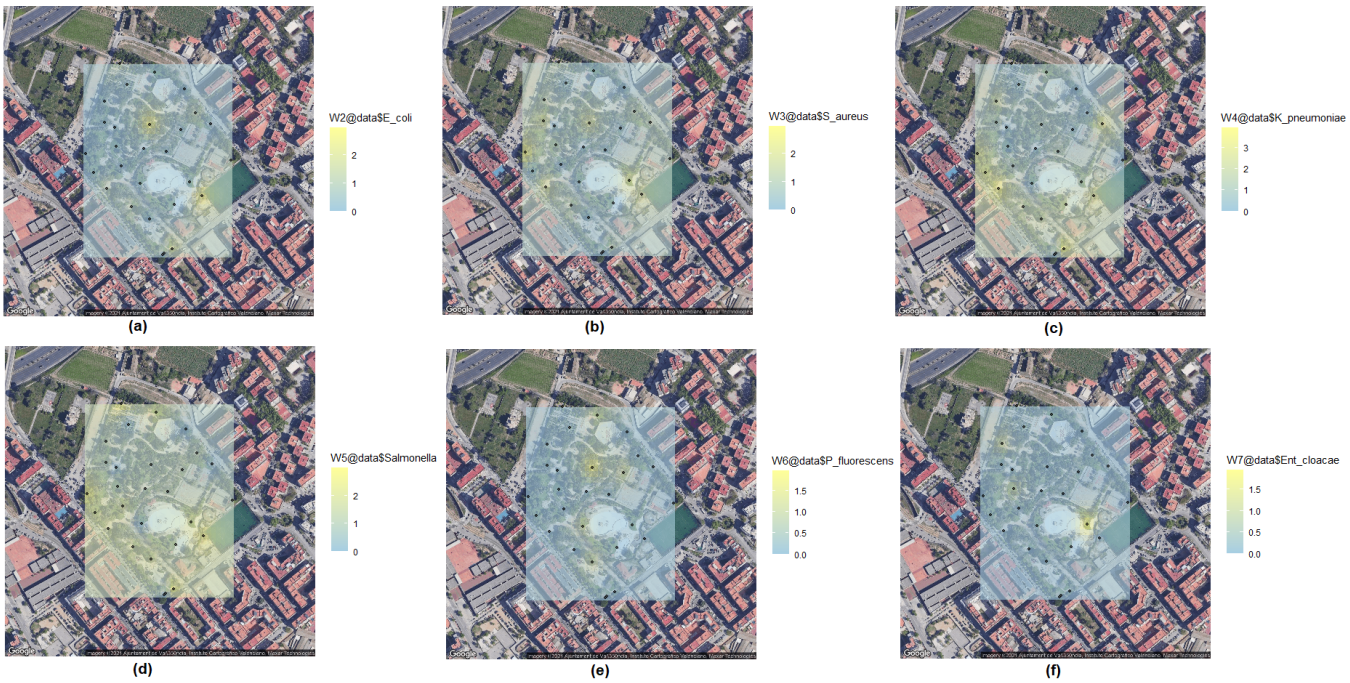

Figure 4. Kriging mapping of inhibition against different pathogens: (**a**) *E. coli*, (**b**) *S.aureus*, (**c**) *K. pneumoniae*, (**d**) *Salmonella* sp., (**e**) *P. fluorescens*, and (**f**) *Ent. cloacae*.

Table 2. Inhibition against relative safe pathogens (number of positive isolates).

Sample	*E. coli*	*S. aureus*	*K. pneumoniae*	*Salmonella* sp.	*P. fluorescens*	*Ent. cloacae*
PKB01	0	0	0	0	0	0
PKB02	0	3	3	3	0	0
PKB03	0	0	0	0	0	0
PKB04	1	1	0	1	0	0
PKB05	0	0	3	2	0	0
PKB06	1	2	4	2	0	0
PKB07	0	0	0	0	0	0
PKB08	1	1	2	2	0	0
PKB09	3	2	3	3	0	0
PKB10	0	1	3	0	0	0
PKB11	0	0	0	0	0	0
PKB12	2	1	4	3	0	0
PKB13	1	1	0	1	0	0
PKB14	1	0	1	1	0	0
PKB15	0	0	2	2	1	0
PKB16	0	0	1	2	1	0
PKB17	0	0	0	1	0	0
PKB18	3	2	1	1	2	0
PKB19	0	0	0	1	1	0
PKB20	0	0	1	2	0	0
PKB21	0	0	2	1	0	0
PKB22	0	0	1	1	0	0
PKB23	1	1	0	3	0	0
PKB24	0	2	1	1	0	0
PKB25	0	1	2	1	0	1
PKB26	0	2	1	2	0	2
PKB27	0	1	0	1	0	1
PKB28	0	0	0	0	1	0
Total	14	21	35	37	6	4

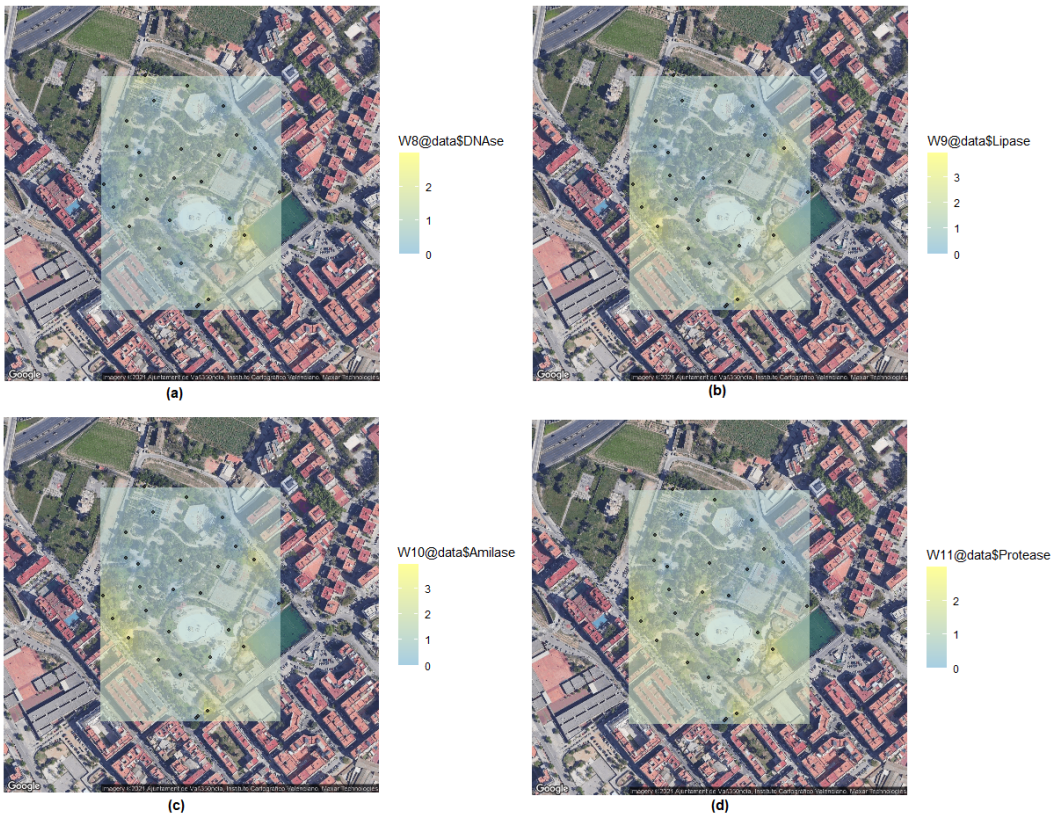

Figure 5. Kriging mapping of positive activities in different cultures: (**a**) DNase, (**b**) lipase, (**c**) amylase, and (**d**) Protease.

Table 3. Positive activities in different media cultures.

Sample	DNase	Lipase	Amylase	Protease
PKB01	0	0	0	0
PKB02	0	3	3	3
PKB03	0	0	0	0
PKB04	1	1	0	1
PKB05	0	0	3	2
PKB06	1	2	4	2
PKB07	0	0	0	0
PKB08	1	1	2	2
PKB09	3	2	3	3
PKB10	0	1	3	0
PKB11	0	0	0	0
PKB12	2	1	4	3
PKB13	1	2	0	1
PKB14	1	0	1	1
PKB15	0	1	1	1
PKB16	1	0	1	1
PKB17	1	0	0	2

Table 3. *Cont.*

Sample	DNase	Lipase	Amylase	Protease
PKB18	1	0	1	1
PKB19	1	0	1	0
PKB20	1	0	1	1
PKB21	1	0	1	1
PKB22	1	0	1	0
PKB23	2	0	1	1
PKB24	1	0	0	1
PKB25	1	2	1	1
PKB26	0	1	1	1
PKB27	1	1	1	1
PKB28	0	0	1	1
Total	10	12	24	19

Typically, a DNase reaction is an indication of pathogenicity for staphylococci and other pathogenic bacteria in clinical assays [34]. However, some soil-isolated bacteria produce transparent halos. Organisms other than staphylococci, *Serratia*, and aeromonads can produce DNase. The presence of extracellular DNA in soil or the availability of plasmid or chromosomal DNA from other soil bacteria that share the same ecological niche can be useful for specialized microbial populations with potential industrial applications, as described previously. [35].

4. Conclusions

Parks are indispensable elements of urban environments. Their soils, and the microbiota that inhabit them, have significant physical, chemical, and biological influences on their characteristics. Thus, urban parks play an important ecological role in preserving microbial diversity, giving the soil the capacity to self-purify pathogenic microorganisms, regulate the greenhouse effect, and perform other functions [36,37]. The ecological functions of the soil are directly linked to the vital activities of bacterial communities that thrive in them. For this reason, the study of these urban parks is of paramount importance.

In this study, we investigated the microbial communities existing in one of the most emblematic urban parks in the city of València. The park is a green space and serves as a recreational area, so microbial exchanges must be frequent. As it is surrounded by busy streets in a completely urban environment, the park's ecological function could be under threat due to continuous use for many generations. To this end, we determined the microbial concentration, particularly bacterial, at various points in the park by conducting a globalized study using the Kriging strategy.

Despite the soils being a mixture of various origins, the amount of bacteria detected is fairly uniform, with no levels higher than 1.20×10^7 in any of the samplings carried out. The population density allowed us to reach a number of global conclusions. On the one hand, in a significant number of sampling points, the isolation of bacteria capable of inhibiting the growth of control microorganisms has been obtained. This led us to believe that the microorganisms that inhabit the park are in intimate contact with the human population. Moreover, the isolated individuals possessed different enzymatic activities, perhaps in response to the environment, allowing them to degrade different compounds of interest.

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Conflicts of Interest: The authors declare no conflict of interest.

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