

# Characterization of bacterial isolates as biopesticides using heat-inactivation preparations in *Culex pipiens molestus*

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## Background

Vector-borne diseases transmitted by mosquitoes has led to tens of millions of infections and millions of deaths annually. Similarly, crop pests also impose devastating effects for economy and food security. In fact, pests account for about 40% of crop yield losses every year. Therefore, control of the vectors and pests has been primarily done using synthetic pesticides. Although useful over several decades, continuous synthetic pesticide use has brought different concerns due to risk of human and animal health, negative effects on beneficial insects long half-life in the environment, and pesticide resistance. The European Commission has provided the "Green Deal" to reduce the use of synthetic pesticides by 50% until 2030 (EU, 2022). There is a current need for other alternatives that are both effective at controlling vectors and pests while encouraging sustainable strategies for the environment.

An alternative to vector and crop pest control could be done using biopesticides. Biopesticides are defined by the United States Environmental Protection Agency (EPA) as natural substances capable of controlling pests. A type of biopesticides can be produced by microorganisms (microbial entomopathogens) (EPA, 2023). To date, the characterization of most biopesticides is limited to either larvicide/adulticide bioassays, but the mode of action, toxin identification, preparation and shelf life remain vastly overlooked. Therefore, the holistic characterization of biopesticides is crucial for the development of new commercial alternatives.

## Goal

The goal of this study was to characterize bacteria collected from different sources in Crete (Greece) regarding larvicidal activity against larvae of *Culex pipiens molestus* using heat-inactivated bacteria preparations. The characterization consisted of a preliminary bioassay screening using live bacteria, bioassays using heat-inactivated bacterial pellets, and the identification of the fraction where the toxin(s) are associated to the bacterial membrane, secreted into the supernatant or both.

## Methods

### 1. Sample collections

Samples were collected from 65 locations throughout Crete (Fig. 1A and 1B.1). Samples were resuspended in 1X PBS and plated in Luria-Bertani and Czapek agar. Then, unique colonies were isolated in fresh LB agar and stored in 1:1 50% glycerol suspension for downstream experiments.

### 2. Live bacteria screening of biopesticides

A subset of isolates was screened as potential biopesticides. The screening was done using 24-well plates. For each isolate, three 3<sup>rd</sup>-4<sup>th</sup> instar larvae were added into 9 mL of distilled H<sub>2</sub>O plus 1 mL of isolate broth. Two wells were tested per isolate. Mortalities were recorded every 24 h for seven days.

### 3. Identification of bacterial isolates by sequencing the 16S rRNA subunit

The identification of bacteria isolates was achieved by sequencing the full-length 16S ribosomal RNA subunit. Identification was assessed by the most probable genus based on the BLASTn against the genome database of the National Center for Biotechnology Information, and building phylogenetic trees. Maximum likelihood trees for each isolate were constructed following the best-fit model with 10000 bootstrap iterations.

## Acknowledgements

This project has received funding from the European Union's HORIZON-CSA (HORIZON-WIDERA-2022-TALENTS-01-ERA Chairs) programme under the grant agreement No 101087110.

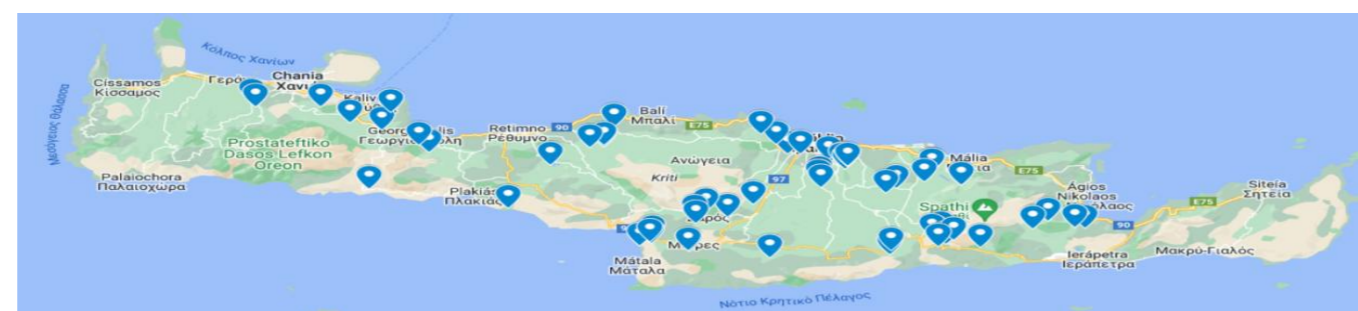
### 4. Bioassays using heat-inactivated bacteria

A single colony per isolate was inoculated in 5 mL of LB broth and incubated for 72 hours at 28°C and 200 rpm in a shaking incubator (Fig. 1B4). Then, samples were incubated in a water-bath for 1 hour at 75°C. LB broth without bacteria was used as a control. Preparations were done in 6-well plates (Sigma-Aldrich, Cat. # CC302) and each heat-inactivated strain was served per well. Briefly, 3 mL of heat-inactivated bacteria were mixed with 3 mL of 4% agar, and 100 mg of ground fish powder.

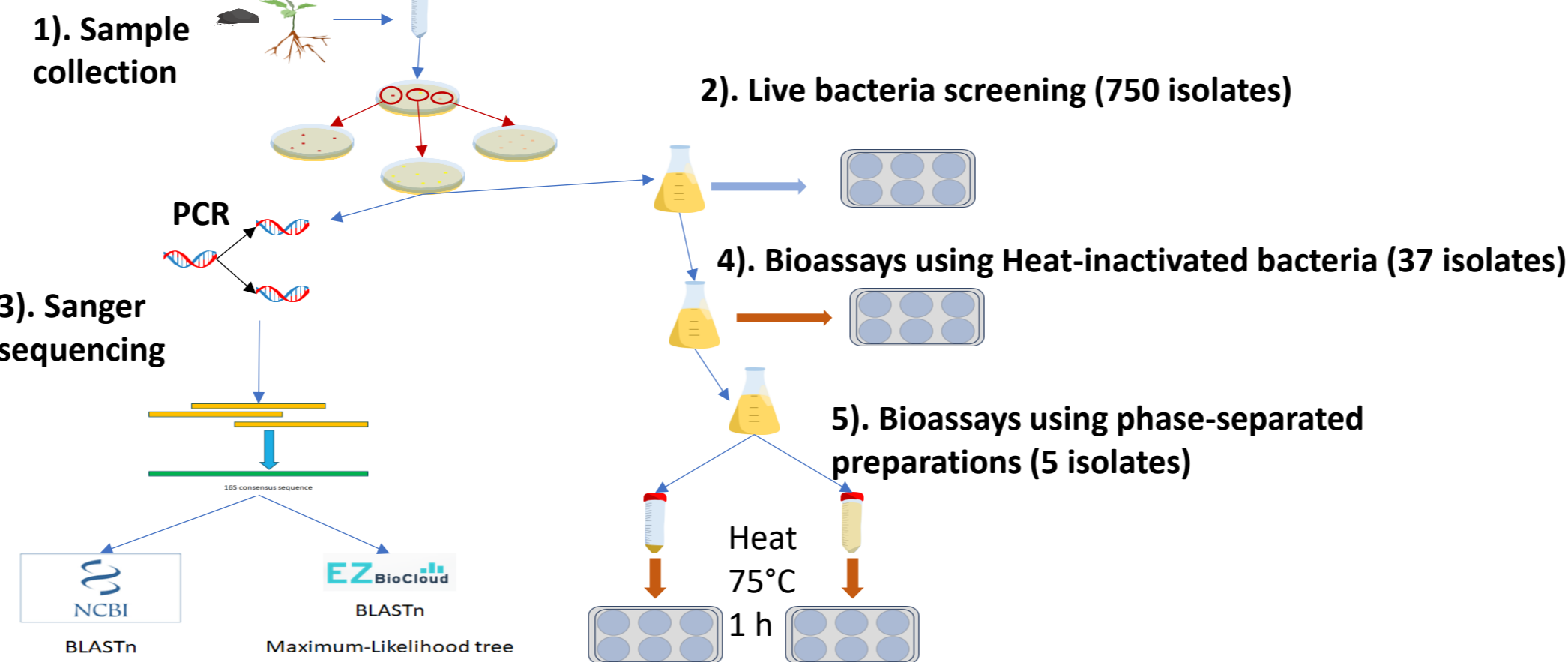
### 5. Bioassays using phase-separation bioassays

A separation method was used to determine whether the insecticidal agents are either secreted, attached into the bacterial cell membrane or both (Fig. 1B5). For each isolate, individual colonies were inoculated in LB broth as previously described and incubated for five days. The inoculates were then centrifuged at 3500 rpm and 10°C for 20 mins. The cell precipitates and supernatants were heat-inactivated in a water bath at 75°C for one hour. Pellets and supernatants were desiccated on a biosafety cabinet model for three days. The desiccates were then crushed into powder and used to prepare the larvicide pellets as previously described.

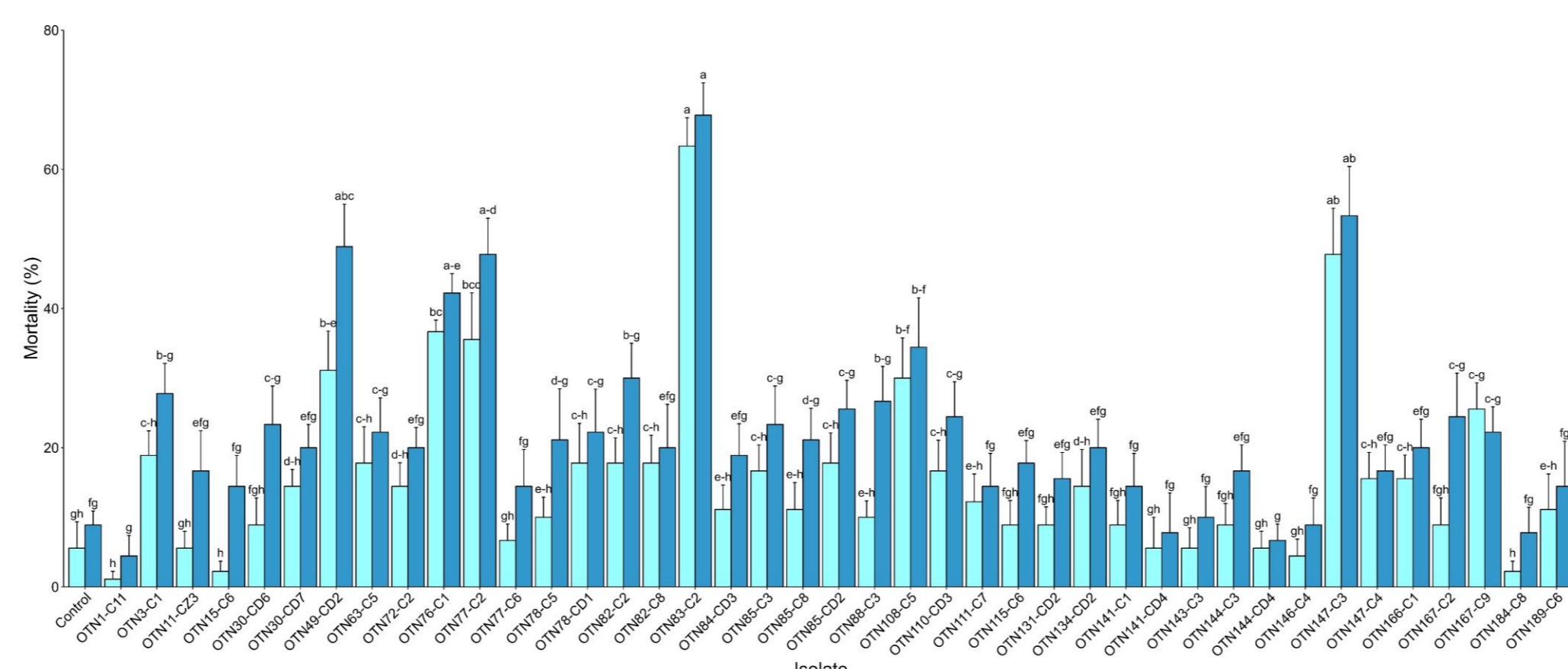
A).



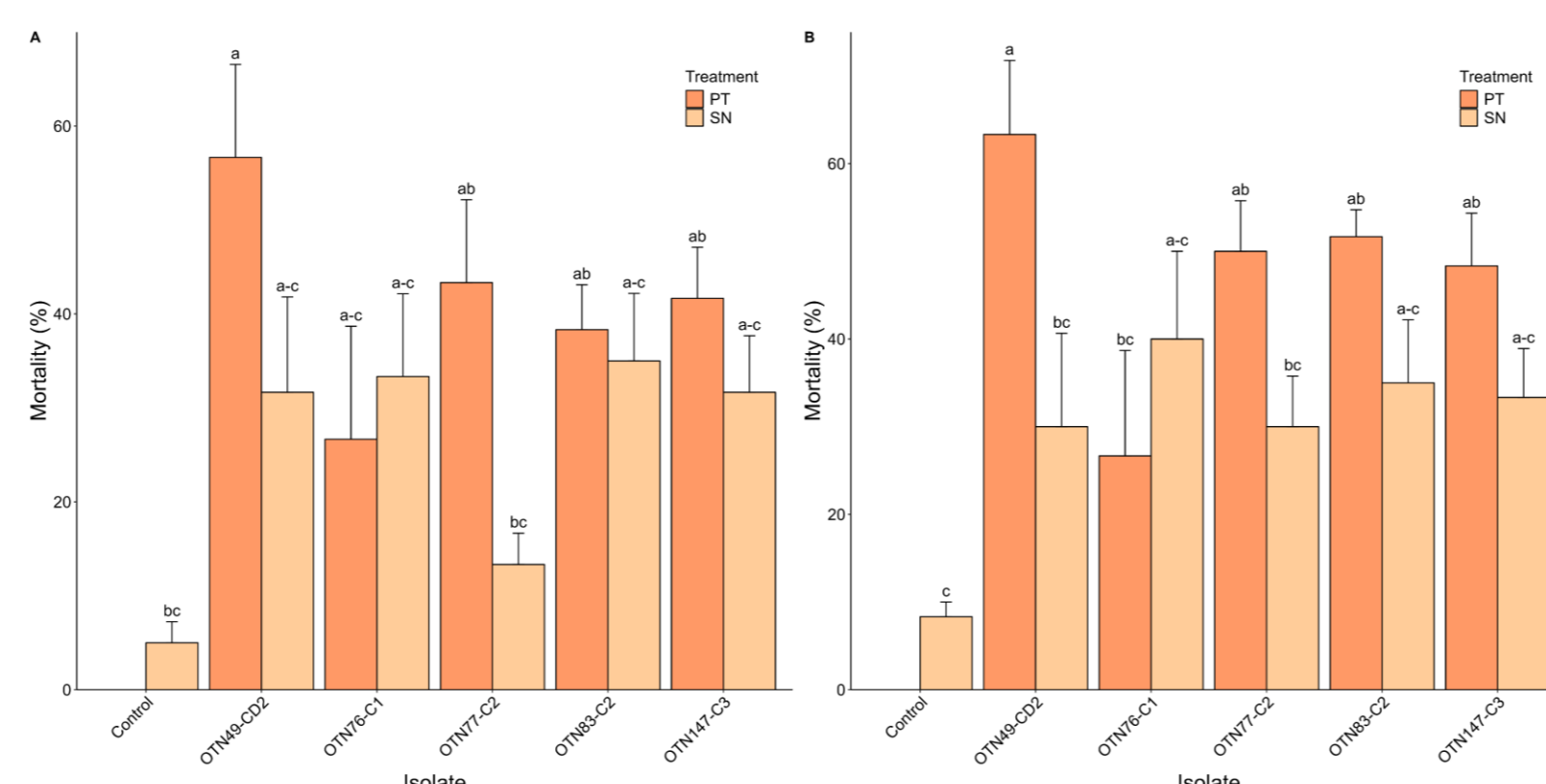
B).



**Figure 1.** Sampling site and project workflow. A). Map of Crete and the 65 locations surveyed. B). Workflow of the project; 1). Sample collection 2). Live bacteria biopesticide screening. 3). Sanger sequencing. 4). Heat-inactivation bioassays. 5). Phase-separation bioassays.



**Figure 2.** Mortality of the top isolates at day 4 (light blue) and day 7 (dark blue) of the bioassays. Bars with different letters indicate significant differences according to the Tukey's test.



**Figure 3.** Mortality of the five top isolates using different preparations as active ingredient (cell-precipitates and supernatants). Panel A and B correspond to day 4 and day 7 of the bioassays, respectively. Bars with different letters indicate significant differences according to the Tukey's test. Pellet preparations are referred as "PT" and "SN" which correspond to cell-precipitate and supernatant, respectively.

## Conclusions

There were five isolates with promising heat-stable activities: OTN49-CD2 (*Pseudoxanthomonas sp.*), OTN76-C1 (*Microbacterium sp.*), OTN77-C2 (*Acinetobacter sp.*), OTN83-C2 (*Bacillus sp.*), and OTN147-C3 (*Micrococcus sp.*).

No significant differences in mortalities between cell or supernatant preparations. Therefore, future preparations will be focused on cell-phase preparations (faster preparation of insecticidal pellets).

## Results

### 1). Sample collection

A total of 1709 isolates were obtained from the 195 samples collected in 2023.

### 2). Live-bacteria screening

A total of 750 isolates were screened. There were 109 isolates that killed 100% of the larvae after seven days. There were 37 out of the 109 isolates that were considered the top biopesticides because they reached 100% by day 3.

### 3). Identification of bacteria

The identification of bacterial genera was successful for the 109 isolates screened. The isolates belong to 37 genera and the most abundant were *Pseudomonas* (21 isolates), *Microbacterium* (11), and *Bacillus* (9). The other genera were *Curtobacterium* (7), *Micrococcus* (7).

### 4). Heat-inactivation

There were significant differences in the mortalities between isolates at day 4 (one-way ANOVA,  $F_{41,336} = 10.31$ ,  $p$  value  $< 0.05$ ) and day 7 (one-way ANOVA,  $F_{41,336} = 7.329$ ,  $p$  value  $< 0.05$ ). At day 7: there were five isolates with the highest mortalities: OTN83-C2 (67.7%), OTN147-C3 (53.3%), OTN49-CD2 (48.8%), OTN77-C2 (47.7%), and OTN76-C1 (42.2%).

### 5). Identification of the source of the toxin(s)

The source of the larvicidal activities were determined for the five isolates that produced highest mortality at days 4 and 7 (Fig. 3A and Fig. 3B, respectively). There were significant differences in the mortalities across preparations and isolates at day 4 (one-way ANOVA,  $F_{11,48} = 5.14$ ,  $p$  value =  $2.81 \times 10^{-5}$ ) and day 7 (one-way ANOVA,  $F_{11,48} = 6.61$ ,  $p$  value =  $1.43 \times 10^{-6}$ ). At day 7, Mortality observed for OTN49-CD2 was significantly higher between the preparations that contained the pellet (63.3%) compared to the supernatant (30.0%) (Tukey,  $p$  value = 0.021). Regarding the supernatant preparations, there were no significant differences between the five isolates and the controls.

Genus	# of isolates
<i>Pseudomonas</i>	20
<i>Bacillus</i>	9
<i>Microbacterium</i>	9
<i>Curtobacterium</i>	7
<i>Micrococcus</i>	7
<i>Stenotrophomonas</i>	6
<i>Chryseobacterium</i>	5
<i>Pantoea</i>	5
<i>Paenarthrobacter</i>	3
<i>Rhodococcus</i>	3
<i>Streptomyces</i>	3
<i>Acinetobacter</i>	2
<i>Dermaococcus</i>	2
<i>Exiguobacterium</i>	2
<i>Kocuria</i>	2
<i>Terribacillus</i>	2
<i>Variovorax</i>	2
<i>Agrobacterium</i>	1
<i>Escherichia</i>	1
<i>Frigoribacterium</i>	1
<i>Herbiconitux</i>	1
<i>Isopentocola</i>	1
<i>Leucobacter</i>	1
<i>Mammaliococcus</i>	1
<i>Mycobacterium</i>	1
<i>Nocardia</i>	1
<i>Paracoccus</i>	1
<i>Peribacillus</i>	1
<i>Planococcus</i>	1
<i>Planomicrobium</i>	1
<i>Pseudoxanthomonas</i>	1
<i>Psychrobacillus</i>	1
<i>Psychrobacter</i>	1
<i>Rheinheimera</i>	1
<i>Serpens</i>	1
<i>Staphylococcus</i>	1
<i>Stutzerimonas</i>	1

**Table 1.** Number of isolates found for each bacterial genus according to the 16S rRNA sequencing.