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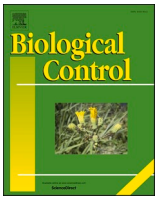

# Assessing the environmental biosafety of phage-based biocontrol applications

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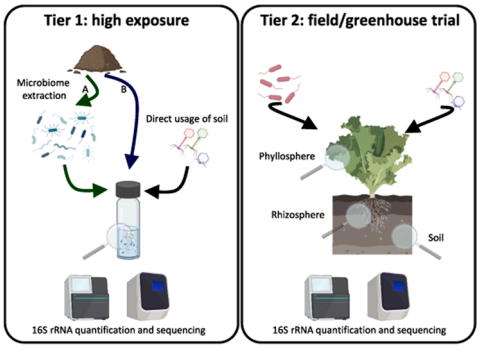
## HIGHLIGHTS

- A pipeline tests safety of phage products in agricultural settings.
- The pipeline can guide assessing environmental safety of phages for EU registration.
- Xanthomonas phages FoX2 and FoX4 do not seem to cause undesired effects on non-target species.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Over the past decades, phage biocontrol as a means of treating bacterial plant diseases has regained keen interest. Indeed, pioneering trials have shown this is a promising strategy to treat different diseases. However, just like other plant protection products, the biosafety of bacteriophages needs to be validated and reported before registration on the European market is possible. In this regard, the EU has provided data requirements for viral biocontrol in EU Regulations 283/2013 and 284/2013. However, the guidelines on how to determine important characteristics with regard to phage biosafety, remain scarce. Based on the current data requirements and literature, we developed a pipeline based on taxonomic analysis using PCR-based 16S rRNA gene amplicon sequencing. As an illustration of the power of this approach, we show that FoX2 and FoX4, capable of infecting and killing *Xanthomonas campestris* pv. *campestris*, appear not to affect non-target species and hence, are environmentally safe.

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## 1. Introduction

During the 20th century, the global population increased by more than fourfold, reaching eight billion today (Roser et al., 2013; United Nations, 2022). This population growth had not been possible without chemical advances in terms of fertilization and protection of crops against plant pests and diseases during the Green Revolution. However, the extensive use of pesticides has had several undesired consequences. Broad-spectrum neonicotinoids, strobilurins/azoles, and streptomycin, for example, led to the development of resistance in insects, fungi and bacteria (Price et al., 2015; Bass et al., 2015; Miller, Ferreira, and LeJeune, 2022; Sundin and Wang, 2018). Moreover, specific chemical pesticides had a detrimental effect on non-target organisms causing a burden on the environment as well as human health (Zikankuba et al., 2019), and have led to a more stringent legislation (Bruce et al., 2017). Today, more and more pesticides are discontinued on the European market. Indeed, EU directives and laws including 91/414/EEC and (EC) 889/2008 led to a withdrawal of many pesticides from the European market over the past decades. At the same time, the rate at which new active substances are introduced in the European market is much slower and withdrawn products often cannot be replaced with equally efficient but environmentally more friendly products (Chapman, 2014). In its ambitious Farm to Fork strategy, the EU aims to reduce the use of chemical and hazardous pesticides by 50 % by 2030 to protect biodiversity. At the same time, the EU acknowledges that new active substances are urgently required to continue agricultural practices and introduced low-risk pesticides, with an emphasis on biological control. These active substances can be introduced to the market by a fast-track authorization procedure and the EU is, therefore, funding several projects in the field of sustainable plant protection (European Commission, 2009).

A large body of literature is trying to understand the potential of bacteriophages, viruses of bacteria, as a biocontrol strategy to reduce the impact of bacterial phytopathogens. Their activity is highly selective towards specific bacterial taxa or even strains, which potentially reduces undesired side effects on non-target organisms and thus makes them an attractive alternative for antibiotics and other chemical plant protection products (PPP) (Hassan et al., 2021). Moreover, the chemical constituents of phages and their degradation products, i.e. proteins and nucleic acids, are non-toxic and easily degradable, which is typically not the case for chemical PPPs. Recent pioneering field trials with phage-based PPPs have, in fact, already shown their potential to replace their chemical counterparts (Buttimer et al., 2017; Holtappels et al., 2021; Stefani et al., 2021; Nga et al., 2021). Although these studies undoubtedly show that phage biocontrol has potential to treat at least some diseases, there are still some key gaps in our knowledge. For instance, phage host interactions might behave differently in complex environments like within microbial communities and soil, in which the presence of potential hosts remains largely unexplored (Wagemans et al., 2022; Koskella et al., 2022).

However, considering that phages, being biological agents, have the potential to increase their environmental abundance due to replication in host organisms, and that environments into which they are released, e.g. soil, are inhabited by natural, complex, highly diverse and not fully characterized microbiomes, there is a challenge to evaluate potential side effects of phage based PPPs. Indeed, bacteriophages could have both direct and indirect effects on a microbiome by kill-the-winner dynamics, resistance development, or mediating horizontal gene transfer, thus driving the evolution of their hosts (Brown et al., 2022). Moreover, by killing their host, they could make nutrients available and alter competition dynamics within an environment (Koskella and Brockhurst 2014; Weitz and Wilhelm 2012). Additionally, phages have been shown to be an important part of the plant microbiome as they influence microbiome diversity (Morella et al. 2018). In doing so, this might cause changes in the functional potential of a microbiome and in the worst case destabilize a microbiome due to the loss of their intrinsic ecosystem services. Considering that earth's microbiomes are indispensable to ecosystem services including nutrient cycling, soil fertility, pathogen and pest regulation, and water regulation and purification, it is of great importance to ensure their protection (Saccá et al. 2017).

In the EU, risk assessment of PPPs is evaluated by advisory bodies like the Environment Agency, the European Medicines Agency (EMA), and the European Food Safety Authority (EFSA). EU Regulations 283/2013 and 284/2013 provide details on the data requirements for registration of active substances. Both documents state that information about the ecological properties, the intended use, survival, reproduction, genetic stability, multiplication, colonization and dispersal of biological control organisms must be provided. However, guidelines on how to determine these characteristics are not available for phages, in contrast to chemical PPPs. Based on these guidelines and available literature, we argue that taxonomic analysis using PCR-based 16S rRNA gene amplicon sequencing from environmental DNA, can provide the best means to assess the influence bacteriophages exert on the microbiomes in PPP receiving environments. To demonstrate the feasibility of this approach, we developed a two-tiered approach with the objective to validate how two *Xanthomonas campestris* pv. *campestris* (Xcc) phages (FoX2 and FoX4) (Holtappels et al., 2021; Holtappels et al., 2022a) affect the soil microbiome, as a case study. Xcc is an important pathogen of crucifers including Brussels sprout, cabbages, broccoli and cauliflower, and causes crop yield decreases of up to 25 % (Holtappels et al., 2022b; Vicente and Holub, 2013; Inagro, 2011). Currently, no sustainable commercial treatments exist and instead, management of the disease relies on prevention by testing seeds for the presence of *Xanthomonas* and by the use of hygienic measures during cultivation.

Also, the extent to which phage biocontrol might affect soil microbiomes remains poorly investigated. However, no general guidelines exist to determine the effect of phage biocontrol. Therefore, we here present a two-tiered approach that can be used to investigate the effect of phage biocontrol on the microbiome. For this approach, phages are first incubated with a microbial cell consortium extracted from the soil to investigate the effect of a continuous high exposure rate on the microbiome. Secondly, the effect of the phages on the microbiome can be investigated by sampling a field experiment.

## 2. Materials and methods

### 2.1. Bacterial strains and phages

*Xanthomonas campestris* strains were isolated from the leaves of symptomatic brassica crops as described previously (Holtappels et al., 2022a). They were plated from glycerol stocks on low salt Lysogeny Broth ($LB_{ls}$) agar and incubated at 25 °C. Resulting colonies were grown in liquid $LB_{ls}$ at 25 °C with shaking (200 rpm). FoX2 and FoX4 were isolated and propagated as previously described (Holtappels et al., 2022b). In short, a bacterial culture of GBBC 1419 or GBBC 1412 was grown to an $OD_{600}$ of 0.3 (corresponding to $1.25 \bullet 10^8$ CFU/ml) and infected (MOI 0.01) with FoX2 or FoX4, respectively. These have been isolated from the soil of infested cabbage fields and their host range was very specific to a subset of *Xanthomonas campestris* pv. *campestris* strains only, as described previously (Holtappels et al., 2022a). The genomes of the bacterial strains can be accessed under BioProject PRJNA729255, the phage genomes under accession codes NC_055836 and NC_055839, for FoX2 and FoX4, respectively. Incubation continued overnight followed by centrifugation (4 °C, 4,000*g*, 1 h) and filtration using Nalgene Rapid-Flow filter units with a 0.45-μm polyethersulfone (PES) membrane. Next, dilutions with the desirable phage concentration were prepared in phage buffer (10 mM Tris · HCl, pH 7.5, 10 mM $MgSO_4$, 150 mM NaCl).

### 2.2. Microcosm experiments

Prior to the field trial, topsoil was collected from the surface 10 cm

from the trial location at Proeftuincentrum voor de Groenteteelt (PCG, Kruishoutem, Belgium; N 50.94337°, E 3.52710°). The soil was a silt loam (10.5 % sand, 77.5 % silt, 12 % clay) with 1.28 % total C and 0.11 % total N content and pH 6.60 in 0.01 M $CaCl_2$. It was sieved (2 mm), mixed, and stored at 4 °C until use.

The protocol to extract bacterial cells from the soil was designed based on Lindahl and Bakken, 1995, Taylor and Williams, 2010, and Dorsch et al., 2012 using shaking instead of harsh physical treatments to improve the survival of more fragile Gram-negative rods, such as *Xanthomonas*, and a Nycodenz cushion to separate the cells from the soil matrix. First, two aliquots of 40 g of soil (dry weight) moistened with distilled water to 50 % water holding capacity were incubated for three days at 16 °C in 500 ml glass bottles sealed with cotton plugs. Then 100 ml sterile, cold (16 °C) phosphate buffered saline (PBS) (8 g/L NaCl, 0.2 g/L KCl, 1.42 g/L $Na_2HPO_4$, 0.27 g/L $KH_2PO_4$, pH 7.4) was added, and the bottles were shaken in a horizontal position on an orbital shaker at 16 °C with 125 rpm for 2 h. The contents of the two bottles were then mixed and allowed to settle for 15 min to sediment the coarse soil particles. The supernatant was distributed to six autoclaved, pre-cooled centrifuge tubes each receiving 20 – 30 ml. A 20 ml 1.3 g/ml autoclaved, cold (4 °C) Nycodenz (Axis-Shield PoC, Oslo, Norway) cushion was pipetted below the extract in each tube. The tubes were centrifuged at 10,000 × *g* for 1 h at 4 °C with a Sigma 4 *K*10 centrifuge with rotor 12165. The supernatant above the Nycodenz cushion was collected into autoclaved, cold (4 °C) Nalgene tubes and diluted in 300 ml sterile, cold PBS. A second centrifugation step of 10,000 g at 4 °C for 1 h followed in a Sigma 6 *K*10 centrifuge with rotor 12500, after which the supernatant was discarded and the cell pellet was resuspended in 40 ml of cold R2B (yeast extract 0.5 g/L; proteose peptone 0.5 g/L; casein hydrolysate 0.5 g/L; glucose 0.5 g/L; starch soluble 0.5 g/L; sodium pyruvate 0.3 g/L; dipotassium hydrogen phosphate 0.3 g/L; magnesium sulfate anhydrous 0.024 g/L). The resulting soil microbial extract was stored at 4 °C and used within one hour to inoculate the microcosms. The viable cell count was determined by plating on R2A (R2B with 15 g/L agar) and the total cell count by fluorescent microscopy with DAPI staining.

The microcosms were set up in 40 ml autoclaved glass vials sealed with cotton plugs. Each microcosm received 18.65 ml R2B and 500 µl 4 mg/ml cycloheximide to inhibit fungal growth. The microcosms were then cooled to 16 °C. At the start of the experiment, each microcosm was inoculated with 750 µl soil microbial extract containing $1.03 \times 10^6$ CFU viable cell count and $2.7 \times 10^7$ total cell count, or 750 µl Xcc GBBC3160 culture in R2B with $10^8$ CFU, or 750 µl sterile R2B. The microcosms were kept at 16 °C in the dark on rotary platform set to 120 rpm. After 26 h of incubation, the microcosms were inoculated with 100 µl FoX4 culture containing $10^8$ PFU, or UV-inactivated FoX4 culture, or sterile phage buffer. At all times, the microcosms were handled within a biosafety cabinet to minimize the chance of contamination.

The microcosms were assigned to nine different treatments, designated as A to I, in three or ten replicates (Table 1). The experiment ran for 504 h (21 days). The microcosms were sampled daily in the first 8 days, and then 11 days, 14 days, and 21 days after their inoculation with the soil microbial extract or Xcc GBBC3160 culture. The first sampling was done immediately before the inoculation with FoX4 or UV-inactivated FoX4. For treatments A-F, the samples were used to measure $OD_{600}$. From treatments G-I, 500 µl samples were frozen at −80 °C until DNA extraction. One replicate of treatment G was lost before the last two samplings. DNA was extracted using the FastDNA Spin Kit for Soil with Lysis Matrix E tubes (MP Biochemicals, Tübingen, Germany). The DNA concentration in the extracts was determined with a NanoDrop spectrophotometer. The DNA extraction failed from one replicate of treatment G and one of treatment H sampled 6 days after inoculation. From three replicates of each treatment at each sampling time, qPCRs were performed to assess the phage concentration and for absolute quantification of the 16S rRNA gene. This whole experimental setup allowed to verify sterility, stability, infectivity and off-target effect of the active phages. The same applies to the inactive phages, while also testing whether any residual nutrients in the (inactive) phage lysate may affect the bacterial cell consortium. The DNA extracts from samples collected 1, 2, 3, 4, 6, 8, and 21 days after their inoculation with soil bacteria (207 DNA extracts in total) were subjected to amplicon sequencing of the 16S rRNA gene.

**Table 1**
An overview of the nine different microcosms from the experimental set up. nine different microcosms were tested in which the effect of phage administration was determined on the soil consortium or on xanthomonas.

| | GBBC3160 | soil bacteria | FoX4 | replicates |
|---|---|---|---|---|
| **A** | – | – | – | 3 |
| **B** | – | – | + | 3 |
| **C** | – | – | Inactive | 3 |
| **D** | + | – | – | 3 |
| **E** | + | – | + | 3 |
| **F** | + | – | Inactive | 3 |
| **G** | – | + | – | 9 |
| **H** | – | + | + | 10 |
| **I** | – | + | Inactive | 10 |

### 2.3. Monitoring the impact of phages in the microbiome during field trials

During the growth season of 2018, two field trials were performed using FoX2 and FoX4 to assess their efficacy against Xcc GBBC 1412 and GBBC 1419 (unpublished field trials), one at PCG and the other at Proefstation Sint-Katelijne-Waver (PSKW, Sint-Katelijne-Waver, Belgium; N 51.078120°, E 4.5281801°). Kruishoutem (PCG) is characterized by a silt loam soil and has a maritime climate with mild winters and cool summers. Sint-Katelijne-Waver (PSKW) is situated more in the center of Flanders, has slightly warmer summers, cooler winters, and a sandy-loam soil type. The amount of rainfall is slightly higher at Kruishoutem. The trial was performed as previously described by (Holtappels et al., 2022b). Three objects were analyzed: (i) the control object which received nor Xcc nor phages, (ii) an object which received Xcc but no phages but instead was sprayed with wetting agent Silwet Gold only, and (iii) an object that received both Xcc and a phage cocktail of FoX2 and FoX4 ($10^8$ PFU/ml), sprayed on the plants together with Silwet Gold. Four plots were sampled from each treatment. To sample the phyllosphere, ~30 cm long sections of the distal part of 6 leaves that were standing vertically, not touching the ground, had no visible sign of rot or other damage, and were from different plants were collected in plastic bags from each plot. Surface soil samples were collected with plastic spoons into 50 ml falcon tubes from three locations in between the middle rows in each plot. To sample the rhizosphere soil one plant per plot was uprooted, the shoot was cut off, and the loosely adhering soil was removed from the roots by shaking before packing the roots into a plastic bag. Rhizosphere samples were not collected from the control object which received nor Xcc nor phages. All samples were transported to the laboratory in cooler boxes.

The leaves were cut avoiding the main vein to collect 15–18 g material in sterile 250 ml glass bottles. 100 ml sterile saline (0.85 %) was added to each bottle before mixing in an overhead mixer with 20 rpm for 20 min at room temperature. This was followed by 1 min sonication and then vigorous shaking. The liquid was transferred from to 50 ml falcon tubes (two per bottle) which were centrifuged for 1 h with 4750 rpm at 4 °C. The supernatant was discarded and the pellets frozen at −80 °C.

To collect rhizosphere samples, the roots were shaken to liberate them from the loosely adhering soil. The root mass was then placed on a plastic tray and fine roots were collected. The collected fine roots were transferred to 100 ml sterile saline (0.85 %) and stirred around gently to wash off debris. The roots were then transferred to paper towels, gently tapped dry, and weighed. They were then put in a 50 ml tube with 30 ml sterile saline and mixed in an overhead mixer for 30 min at 4 °C with 10 rpm. The solution was transferred to two 15 ml tubes and centrifuged for 40 min with 4750 rpm at 4 °C. The liquid was discarded and the pellets

were frozen at −80 °C.

Total DNA was extracted from the phyllosphere and rhizosphere cell pellets and from 0.5 g soil from the surface soil samples using the FastDNA Spin Kit for Soil with Lysis Matrix E tubes with the objective to utilize it in qPCR reactions for the quantitative detection of the applied phages and for characterization of the bacterial community structure by 16S rRNA gene amplicon sequencing using the Illumina MiSeq technology.

### 2.4. qPCR protocol

qPCRs were performed using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo scientific). Primers for FoX2 and FoX4 were designed according to Pritchard and colleagues (Pritchard et al., 2012). In short, primers were predicted across the phage genomes using Primer3 after which primers that did not amplify within coding sequences were removed (Rozen and Skaletsky, 2000). Next, primer sets were tested in silico using PrimerSearch after which only primer sets that amplified fragments of 100 bp without cross-amplification were retained (Rice et al., 2000). To avoid amplification in presence of non-target species, a BLAST search was performed using the remaining primers. Eventually, the forward and reverse primer sequences for FoX2 were 5′-GATGGACCGAAACAGGATCT-3′ and 5′-ATCTTGGAAGACGGCATTTC-3′. For FoX4, the sequences were 5′-ATGATCTCTGGGCGGGTACTC-3′ and 5′-GTTCGTCAGTTCGTTGCCTA-3′. Reactions were performed with a StepOnePlus Real Time PCR System (Life Technologies GmbH, Darmstadt, Germany) in a final volume of 25 µl with 300 nM of each primer. An initial denaturation step of three minutes at 95 °C was followed by 40 cycles of denaturation (95 °C, 15 sec) and annealing/amplification (60 °C, 60 sec), followed by a melt curve analysis. The reaction efficiency was always higher than 95 %, every signal above the threshold of detection was considered a positive result.

For bacterial quantification, the Maxima Probe qPCR ROX master mix was used, as previously described (Szoboszlay et al., 2019). 500 nM of each primer was used along with 200 nM of the FAM-labeled TaqMan probe. A total of 2 µl of template DNA diluted 50-fold in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8) were used in each 20 µl reaction. The forward and reverse primer in these reactions were 5′-ACTCCTACGGGAGGCAG-3′ and 5′-GACTACCAGGGTATCTAATCC-3′, respectively. The probe was 5′-TGCCAGCAGCCGCGGTAATAC-3′. Reactions were performed under the same conditions as for the phage quantification.

### 2.5. Library preparation, DNA sequencing and data processing

The V4 region of the 16S rRNA was amplified for taxonomic analysis of the samples following the protocol of Kozich et al. (2013) with primers 515f (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806r (5′-GGACTACNVGGGTWTCTAAT-3′) (Walters et al., 2016). In case of the phyllosphere samples, the number of PCR cycles was increased to 35, and to avoid amplification of the 16S rRNA gene from mitochondria and chloroplast, mPNA and pPNA (PNA Bio Inc, Thousand Oaks, California) PCR blockers were added to the reactions in 0.5 µM. The PCR products were purified with the HiYield PCR Clean-up & Gel-Extraction kit (SLG) followed by quantification with a Quant-iT PicoGreen dsDNA assay (Invitrogen, Darmstadt, Germany). Equimolar amounts of the purified PCR products were pooled for paired-end sequencing on a MiSeq instrument (StarSEQ - Mainz, Germany). The samples from the field trial were sequenced with the V2 chemistry and 2 × 250 bp read length while the microcosm samples were sequenced with the V3 chemistry and 2 × 300 bp read length. Reads were obtained from StarSEQ in the cassava 1.8 format and processed with QIIME2 version 2022.11 (Bolyen et al., 2019). First, the reads were filtered with QIIME2's DADA2 plugin (2022.8.0) (Callahan et al., 2016). Instead of rarefaction, we used scaling by ranked subsampling with the QIIME2 SRS plugin (2021.4.0) (Beule and Karlovsky, 2020). To quantify α-diversity, the Simpson and Pielou index were calculated. β-diversity was inferred by calculating the Bray-Curtis distance between samples. Phylogeny was determined using the Fasttree protocol (version 2022.11.1). Amplicon sequence variants (ASV) were classified based on the SILVA reference release 138.1 (Pruesse et al., 2007) using the QIIME2 plugin RESCRiPT (2022.8.0 + 1.g2abcc90; Robeson et al., 2021). Statistical tests were also performed in QIIME2 using the default options, a pairwise Kruskall-Wallis test ($\alpha = 0.05$) for the α-diversity metrics and pairwise PERMANOVA with 999 iterations for the β-diversity. Data from QIIME2 were exported as csv, and R (version 4.2.2) was used to create all figures using the ggplot2 package. Additionally, ALDEx2 (version 1.30.0) was used in R to perform a differential abundance analysis. For the microcosm experiment – in which the diversity was relatively low – species were merged at the genus level using the function "tax_glom", prior to analysis with ALDeX2. ASVs were considered differentially abundant when the Benjamin-Hochberg corrected p-value of the Wilcoxon rank rum test was lower than 0.05. For the field trial – in which diversity was much higher – species were merged at the class, order, family and genus level, prior to analysis. ALDeX2 was used at each of these levels separately, with the same significance criteria as for the microcosm experiment.

### 2.6. Visualization and analysis of other data

The graphs of the ODs and qPCRs were generated with JMP Pro 16 (SAS Institute Inc., Cary, NC, 1989–2021). Statistical analyses were performed using the Tukey-Kramer test at an α level of 0.01. Before this test was performed, data normality of all objects was assessed by fitting a normal distribution and testing the goodness of this fit by means of a Shapiro-Wilk test. Homoscedasticity was tested by means of the O' Brien test.

## 3. Results and discussion

### 3.1. A conceptual approach for phage risk assessment

Plant pathogenic bacteria have diverse interactions with their plant hosts. For instance, they can colonize the above ground parts including the leaves, stem or fruits, colonize the inside of their host or remain at the rhizosphere. As of today, different strategies are used in phage biocontrol depending on the pathosystem including seed, spray and drench treatments (Holtappels et al., 2021). Regardless of the strategy used, though, surface soil and rhizosphere will be most likely to obtain a high phage concentration for a relatively long time. This is due to inactivation of phages in the phyllosphere (e.g. desiccation or UV), and because to run-off caused by rain or irrigation (Iriarte et al., 2007). Bearing this in mind, a two-tiered approach was developed, shown schematically in Fig. 1.

The first tier consists of the incubation of a microbial cell consortium with a high and steady concentration of the phage PPP. This straightforward approach allows the phage concentration to remain high due to the absence of abiotic factors that might cause virion inactivation. In addition, this set-up is relatively cheap, allowing to include a high number of repeats for good statistical power and unforeseeable events can be circumvented (e.g. extreme drought or floods). Finally and most importantly, soil particles to which the phages might adsorb are not present. Hence the effective concentration to which the members of the soil community are exposed equals the concentration of phage administered. This great control of phage exposure allows to study any potential hazard the phage product can cause. In case this tier would give indications that microbiomes are affected in their composition, and that one of the reasons could be linked to the killing of specific community members, e.g. those closely related to the target organisms, it could make sense to add another level of complexity to this tier, e.g. by incubating the phage-based product directly with soil samples instead of soil suspensions. On the one side, this level would allow to also include members of the soil microbiome which could have been missed by the

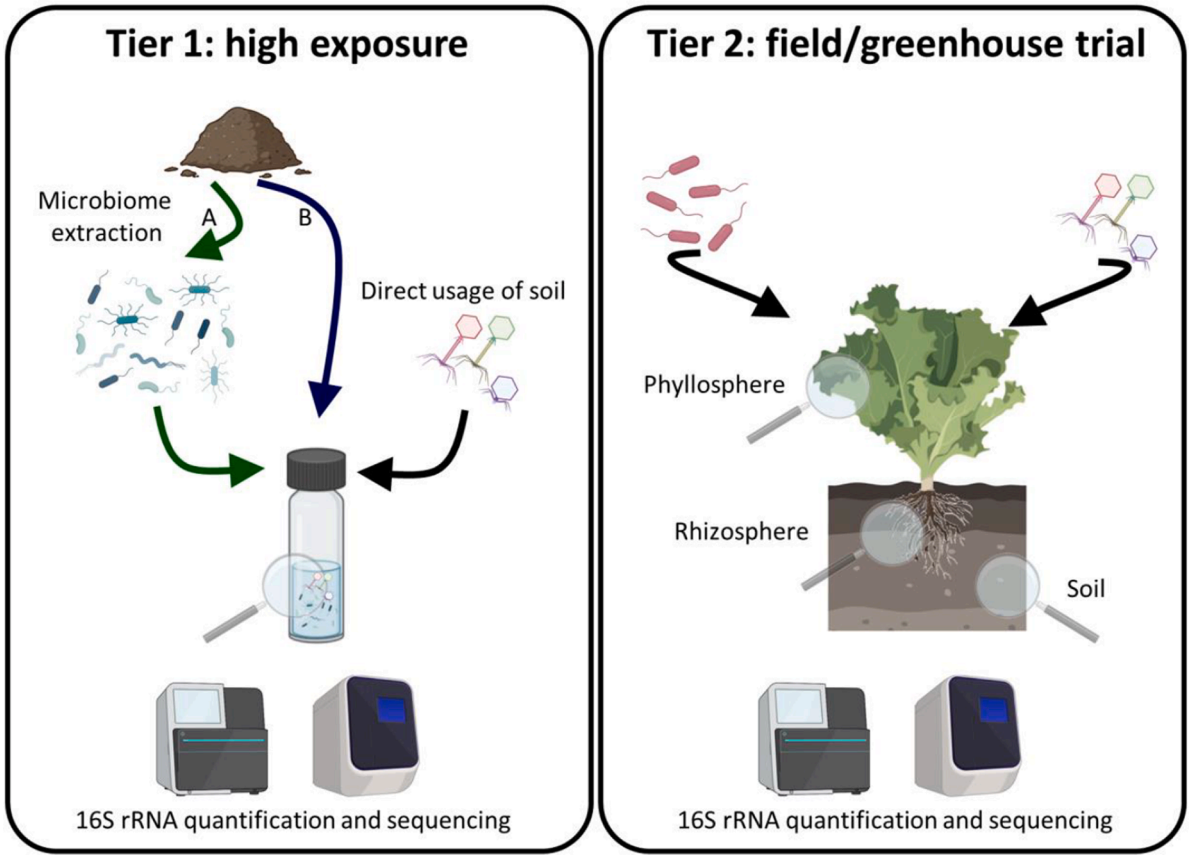


**Fig. 1.** Schematic overview of a proposed two-tiered approach to assess the effect of phages on the microbiome. Tier 1 allows to determine the effect of constant high phage exposure. This can be done by extraction of the microbial cells or including soil to test its shielding effect against the phage exposure. Tier 2 consists of a set-up in which the phage treatment is tested in a relevant set-up. Depending on the pathosystem studied, this can be done in field experiments or greenhouse trials. (Created with BioRender.com).

soil extraction approach. On the other hand, viruses can adsorb to organo-soil particles by complex mechanisms, depending on virus type and kind of soil particle among others (Bellou et al., 2015; Katz et al., 2018; Bi et al., 2022). Today, the role of virion adsorption in their inactivation is unclear (Sykes and Williams, 1978; Bellou et al., 2015). Thus, including soil particles might reveal whether they can reduce or avoid any off-target effects, if any are observed. This extension of Tier one may not be needed if no effect is observed, unless it is suspected that potential hosts of the phages have not been extracted from the soil. On the other hand, if some non-target organisms are affected during Tier one, this set-up allows to determine if this is also true under more realistic environmental conditions.

Tier two consists of representative field trials during which the phage's efficacy is investigated. Sampling from the crop's phyllosphere, rhizosphere and the surrounding surface soil allows to determine the effect of the phage product on members of the different niches in the environment and also estimate the environmental persistence of the PPP itself. This approach would always be advisable but depending on the risks identified during tier one, the intensity and level of monitoring can be adjusted.

### *3.2. FoX4 remains active in the microcosm and affects solely its host's biomass*

First, the ability of the FoX phages to infect their host in the tier one microcosm setup was investigated. For this purpose, the OD of microcosms only containing Xcc and phage, inactivated phage or a negative buffer control (Table 1, treatment D-F) was measured over time. As a control for sterility, microcosms with only phage, inactivated phage or a negative buffer control (Table 1, treatment A-C) were also monitored over time, showing no growth. As shown in Fig. 2A, for the samples containing Xcc, the bacterium was able to grow well in the microcosm medium, achieving the stationary phase after 50 h. Adding UV-treated phages did not affect the growth of Xcc confirming their full inactivation. However, active phages remained active in the microcosm medium. Indeed, a growth delay of about 50 h was observed, after which resistance emerged and the population began increasing again. Interestingly, the maximal OD reached was significantly lower for the treatments with active phages. This could mean that resistant Xcc needs more energy to sustain their biomass. However, since we focused on validating the ability of FoX4 to infect in this medium, this was not investigated further.

In similar fashion, we tested the effect of active and inactive phages on the growth of the soil bacteria. We tracked the evolution of the total count of the 16S rRNA gene over the course of the experiment for microcosms containing the soil bacterial consortium and phage (active, inactive, negative buffer control) (Table 1, treatment G-I). As can be observed from Fig. 2B, the total biomass increases over time and reaches a steady state, lasting from about 150 to 350 h after incubation, after which it begins to decline. No differences in biomass are observed for any of the conditions showing that FoX4 does not affect the overall growth of the biomass, neither does it affect its decline. The qPCR confirmed that FoX4 remained present at high concentrations during the entire experiment (Fig. 2C). The Tukey-Kramer test (all P-values $> 0.03$) showed no significant differences between any of the timepoints, suggesting that the phages were unable to propagate on the soil microbiome, neither did their concentration decline over time. qPCRs of the treatments where no phages or UV-inactivated phages were added never

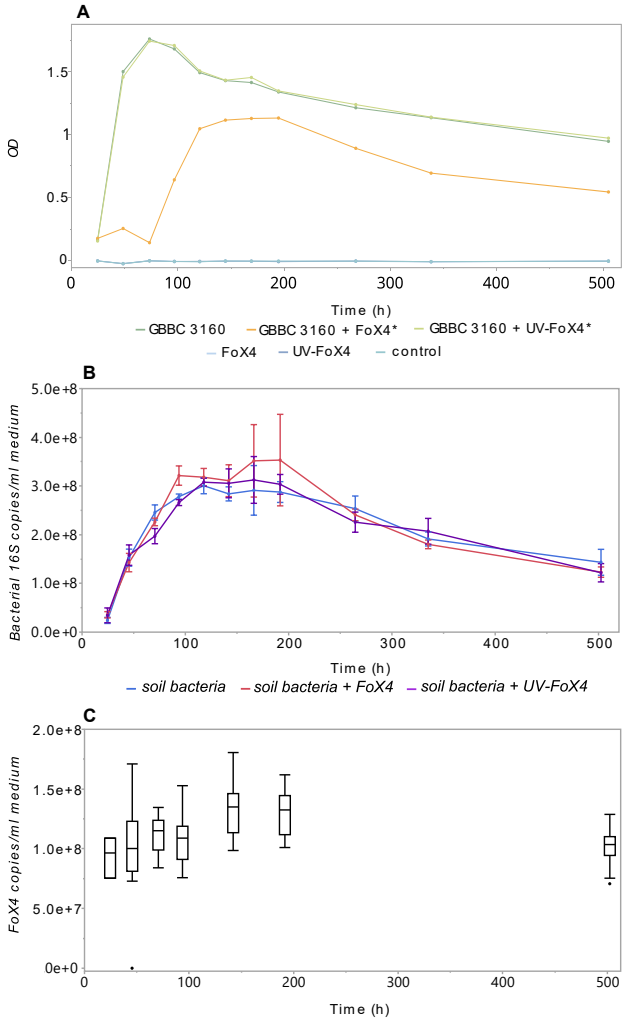


**Fig. 2.** A high concentration of FoX4 only affects it host. (A) Growth curves of Xcc in microcosm medium show that Xcc growth is only affected by active phages. Active phages increase the time for Xcc to achieve its stationary phase, which has a lower OD compared to the conditions without phages or with UV-inactivated phage. (B) Neither active phages nor UV-inactivated phages affect the biomass of soil bacteria in microcosm medium. (C) Phages remain present at high concentrations throughout the experiment in microcosms with soil bacteria and active phage. No samples were taken between 200 h and 500 h.

had detectable numbers of phages and hence, are not shown.

### 3.3. The soil microbial community does not respond to phage FoX4 in absence of its host

To test whether the soil bacterial community was affected by the presence of FoX4, we sampled the microcosms that received soil bacteria and phage, inactive phage, or buffer in absence of Xcc (Table 1, treatment G, H and I). Samples were taken from all ten replicates from these treatments at the following days after start: 1 d, 2 d, 3 d, 4 d, 6 d, 8 d and 21 d, respectively. Hence, the analysis included 207 samples accounting for a total of 380 ASVs. Pielou's evenness indices and Simpsons diversity index were computed (Fig. 3A and B). Both the evenness and richness increased during the incubation of the microcosm. However, the three treatments never differed at the same timepoint (Kruskal-Wallis, all p-values $> 0.22$). As such, there is no indication that FoX4 affects the α-diversity.

Similarly, β-diversity was assessed by calculating the Bray-Curtis distance between the samples, as shown in the Principal Coordinate analysis (PCoA)-plot (Fig. 3C). Over time, the microbial composition changed. Additionally, the dissimilarity between samples increased over time but also here, there is no indication that FoX4 affects the $\beta$-diversity at a given timepoint (PERMANOVA, all p-values $> 0.397$). The change in microbial composition is also reflected in the relative abundance plot (Fig. 3D). Proteobacteria and Firmicutes initially represent the most dominant phyla in the microcosms. However, as time elapsed, the relative abundance of Bacteroidetes increased becoming as dominant as the Proteobacteria, while the Firmicutes vanished almost entirely. At the genus level, *Pseudomonas* was the most dominant at the start of the experiment, making up for more than 70 % of all ASVs. As the experiment proceeded, the dominance of *Pseudomonas* decreased and eventually, *Pseudomonas* and an unclassified *Enterobacteriaceae* genus became the most dominant with a similar presence of around 15 to 20 % of the ASVs each. *Bacillus* were the most dominant Firmicutes at the beginning of the experiment, accounting for 25 to 32 % of the ASVs. At the end of the experiment, *Bacillus* made up less than 1 %. None of the Bacteriodetes made up for less than 0.2 % of the ASVs at the start of the experiment. At the end of the experiment though, the share of *Chryseobacterium*, *Sphingobacterium*, and *Pedobacter* increased, each reaching 23 to 28 %, 8 to 9 % and 6 to 7 % of the ASVs, respectively.

Since the β-diversity did not show whether individual members of a community were affected, we performed an analysis with ALDeX2, comparing the different treatments at each timepoint. No differentially abundant ASVs were identified, suggesting that FoX4 did not affect any of the members of the microcosms, or the effect was too small to be detected. An important note to make, is that none of the ASVs was assigned to members of the *Xanthomonas* genus, indicating that there were no natural occurring phage hosts present in the samples. Therefore, it makes sense that FoX4 had no effect on the soil microbiome. A different result might have been obtained if natural occurring hosts of FoX4 were present in the samples. A justified question is whether it would really be a risk if a phage affected such closely related members. To test this, we suggest isolating and sequencing closely related members from the microcosm to check if they are affected by a phage of interest. This would allow to confirm whether this member is also a phytopathogen or whether it is harmless to the plant. In the second case, it might be better to consider using a more specific bacteriophage.

### 3.4. FoX2 and FoX4 can persist in a field environment

To be able to link potential changes of the environmental microbiomes to phage treatments, the abundance of the phage and their infectivity needed to be quantified under field conditions. This was evaluated in a field trial at two different sites in Belgium. At each site, the following treatments were assessed and compared: a treatment without Xcc and no phage product, another treatment with Xcc but no phage and finally also a treatment with Xcc and the phage product.

First, the phage concentrations were determined in the phyllosphere, soil and rhizosphere, using qPCR. Samples of leaves that had not received phages always showed negative qPCR results. Leaves treated with phage showed positive qPCR values for both phages, except for the leaves from PCG, where FoX2 could not be retrieved. This was promising given the fact that phage inactivation in the phyllosphere due to UV is regarded as a major limitation for phage biocontrol applications under open field conditions (Jones et al., 2007; Born et al., 2015; Balogh et al., 2018). Surface soil and rhizosphere samples were always negative for FoX4, except for one sample in PSKW. FoX2 on the other hand could be detected in the surface soil in PSKW but not PCG. An overview of the samples in which the phages were detected is provided in Table 2. Interestingly, samples from plots where no phage cocktail was sprayed sometimes had positive qPCR values, suggesting a cross contamination in the field plots. However, this does not necessarily indicate that the dispersion of FoX2 is problematic for environmental safety. Indeed, future commercial phage products will likely be used to treat entire fields preventively. A phage capable to spread and achieve high

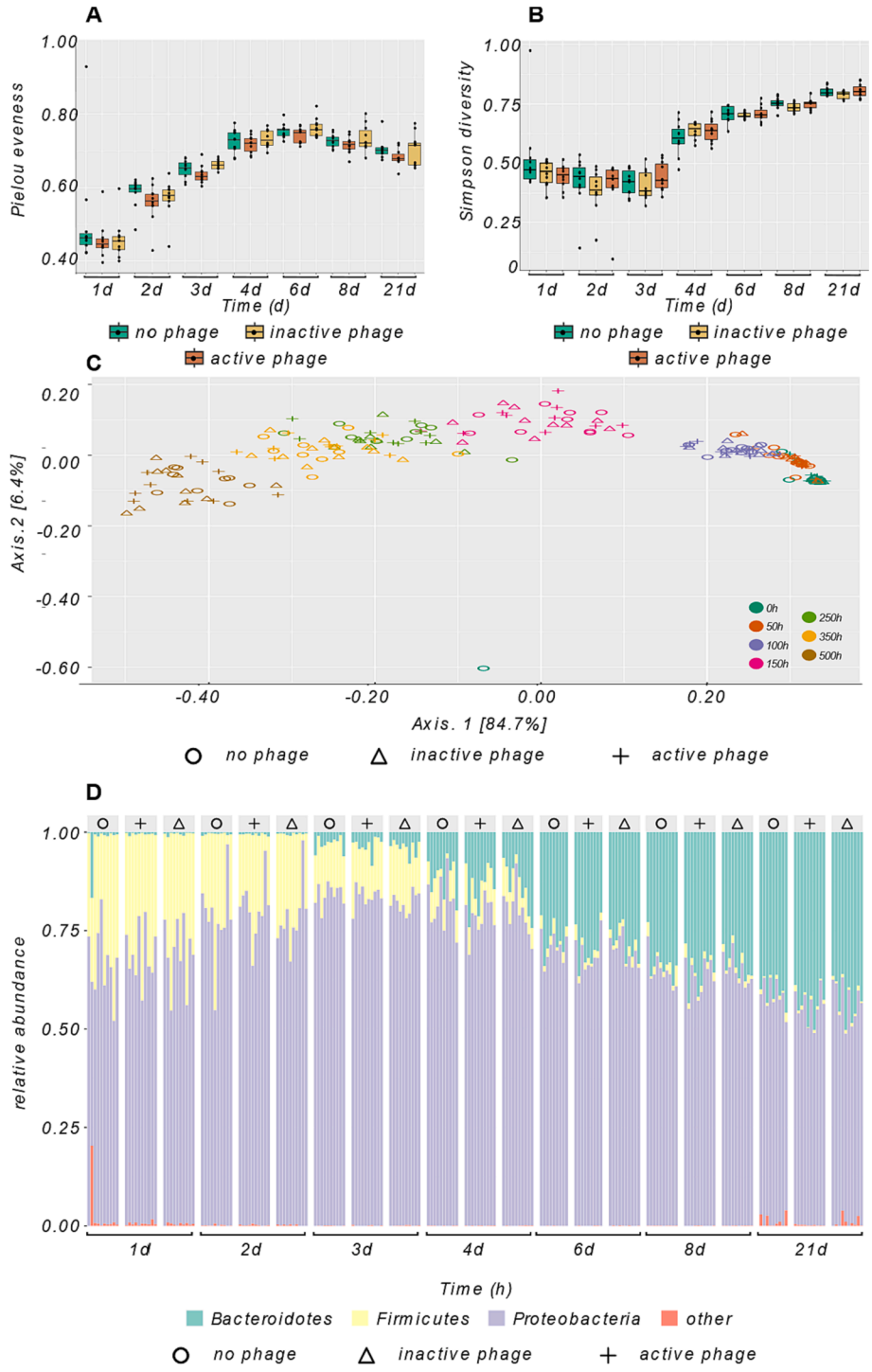


**Fig. 3.** Diversity of the microcosm of treatment G-I. Addition of phages or UV-inactivated phages influences neither evenness nor richness (A, B), neither does it affect dissimilarity between the sample (C) or species composition as shown in the taxa barplot and confirmed with ALDeX2.

concentrations at a place where its host is present (auto-dosing ability) is desirable for successful treatment. However, for future risk assessments, it might be useful to retain a bigger buffer zone between different plots allowing to determine whether a phage can disperse. This can be achieved by dividing a test field in plots in which adjacent plots are used to cultivate different crops, that cannot be infected or colonized by the studied pathogens.

### 3.5. Response of the soil community to FoX2 and FoX4

In context of the two field experiments, we analyzed the composition of microbiomes from soil, rhizosphere and phyllosphere, since the presence of the phage product in these microhabitats was expected. In total, 24,950 different ASVs were obtained from the samples of the field trial. A total of 8,193 ASV were obtained from the surface soil samples of PCG. Hence, we were able to extract and cultivate around 4–5 % of the bacteria present in our microcosm, which had a total of 380 ASVs. Similarly, the ASVs in the microcosm experiment could be assigned to

**Table 2**
Summary of the samples where FoX2 and FoX4 were detected. Crossed cells represent objects that were not sampled. Grey cells represent samples where no detectable amount of phage was measured, while samples with check marks (✔) represent samples where phages were present at a detectable amount. Red values represent samples where no phages were added, blue values represent samples where phages were added. The qPCR limits were as follows: 256 copies/g (d.w.) for leaf samples, 16,134 – 31,842 copies/g (d.w. surface) soil and 1,925 copies/g (d.w.) for roots.

| **FoX2** | Sample type: | Leaf | | | | Surface soil | | | | Rhizosphere | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Replicate: | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| PCG | No Xcc, no phage | | | | | | | | | | | | |
| | Xcc, no phage | | | | | | | | | | | | |
| | **Xcc, phage** | | | | | | | | | | | | |
| PSKW | No Xcc, no phage | | | | | ✔ | ✔ | ✔ | | | | | |
| | Xcc, no phage | | | | | ✔ | ✔ | ✔ | ✔ | | | | |
| | **Xcc, phage** | ✔ | ✔ | ✔ | ✔ | | ✔ | ✔ | ✔ | | | | |
| **FoX4** | Sample type: | Leaf | | | | Surface soil | | | | Rhizosphere | | | |
| | Replicate: | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| PCG | No Xcc, no phage | | | | | | | | | | | | |
| | Xcc, no phage | | | | | | | | | | | | |
| | **Xcc, phage** | ✔ | ✔ | ✔ | ✔ | | | | | ✔ | | | |
| PSKW | No Xcc, no phage | | | | | | | | | | | | |
| | Xcc, no phage | | | | | | | | | | | | |
| | **Xcc, phage** | ✔ | ✔ | ✔ | ✔ | | ✔ | | | | | | |

286 different taxa, while in the soil of PCG, the ASVs were assigned to 1,716 taxa. Indeed, only a few percent of the bacteria can be cultivated in lab conditions, emphasizing that field trials are important for testing a broad range of different members of the soil microbiome (You et al., 2022; Wade, 2002).

Evenness and richness across the samples were computed (Fig. 4A and B). For both sites, surface soil samples had the highest diversity and evenness, followed by rhizosphere and phyllosphere samples (all adjusted p-values $< 0.004$). However, no significant differences were observed between samples of the same origin of PCG and PSKW. When comparing the diversity and evenness across the different treatments, adding Silwet Gold and Xcc or Silwet Gold, phage and Xcc, there is no indication that these have affected the microbial composition.

Each type of sample was characterized by a specific community, as shown in Fig. 4C. Interestingly, comparison of the phyllosphere, rhizosphere and surface soil samples between both sites revealed that the communities were significantly different (all adjusted p-values $< 0.001$). However, the effect of the treatment did not significantly affect the microbial composition (Fig. 4D). Hence, there is currently no indication that FoX2 and/or FoX4 might affect non-target species. *Xanthomonas* ASVs were detected in PSKW surface soil, PSKW rhizosphere and PSKW phyllosphere. This was true for the control plots, the plots that received Silwet and Xcc and the plots that received Silwet, Xcc and phage. *Xanthomonas* ASVs in PCG were also found across these environments. However, except for one phyllosphere sample in the control plot, none of the samples from the control plots had *Xanthomonas* ASVs. Therefore, it seems possible that the absence of *Xanthomonas* ASVs in the microcosms is due to the absence of this genus in the surface soil of PCG, rather than due to an inability of our protocol to extract the microbial soil consortium. Just like in the microcosm experiment, ALDeX2 was used to check for differentially abundant ASVs. This was done after agglomerating species at the class, order, family and genus level, respectively. However, no differentially abundant ASVs were identified at any of these levels.

One important note though is that 2018 had particularly high temperatures and low precipitation. Indeed, the growing season for cabbage had average temperatures of 2 °C higher on average. In July, in the middle of the cultivation season, this was even 3.6 °C. At the same time, the average precipitation was 24 % lower and there were severe droughts, especially in July and August. Finally, 23 % more sunshine hours were observed (MeteoBelgie, 2019). Therefore, it is highly likely that Xcc, which naturally infects plants by entering the hydathodes through guttation droplets has been hampered in its ability to successfully infect the crops by this weather (Vicente and Holub, 2013). Indeed, no symptoms were observed at PCG and only very mild symptoms were observed at PSKW. This might explain why, even though ASVs classified as *Xanthomonas* were present at detectable levels in the microbiome in our field trial, their presence seems not to have affected the microbial composition. In contrast, a previous trial with Xcc on winter oil rapeseed did show its decreasing effect on the richness and composition of the phyllosphere (Jelušić et al., 2021).

Previously, the effect of *Ralstonia solanacearum* phages on the microbiome of tomato was investigated in a greenhouse and field trial (Wang et al., 2019). Here too, no off-target effects were observed for the administered phages. The authors showed that *R. solanacearum* had the ability to shift the community composition of symptomatic plants. This effect could be reduced using phage biocontrol, probably by making the pathogen less competitive. In doing so, Wang and colleagues have shown that, at least in some cases, phage biocontrol might be beneficial for the plant's microbiome. Although we have been unable to determine if FoX2 and FoX4 have a protective effect on the microbiome, we have shown that they do not seem to have any off-target effect, which was the priority of this experiment.

### 3.6. Concluding remarks and future perspectives

*X. campestris* is an important plant pathogen for which there are currently no sustainable commercial treatments. Disease management currently focusses on prevention and using certified seed. However, outbreaks of black rot are still occurring and hence more tools are required to prevent them. Previously, the efficacy of *Xanthomonas*-specific phages was already shown (Holtappels et al., 2022a). However, the effect of the phages on the naturally residing bacterial communities remained to be elucidated. Therefore, we developed a two-tiered setup to that can be used as a state-of-the-art approach to test safety of phage products. In this work, we have shown that there is no indication that the application of FoX2 and FoX4 results in a tangible effects on the composition of microbiomes present in receiving environments, even if highly sensitive detection methods are applied. Thus for this particular case we can conclude that there are no indications yet that our phage based product has an adverse effect on non-target organisms, as has been hypothesized previously (Buttimer et al., 2017).

To our knowledge, there have been only a very limited number of studies focusing on the effect of phage biocontrol on the microbiome together with the one of Wang and colleagues (Wang et al., 2019). The two-tiered approach introduced here can be used to study the effect of phage biocontrol for other pathosystems in a standardized way. The insights from future studies will be important to get a deeper

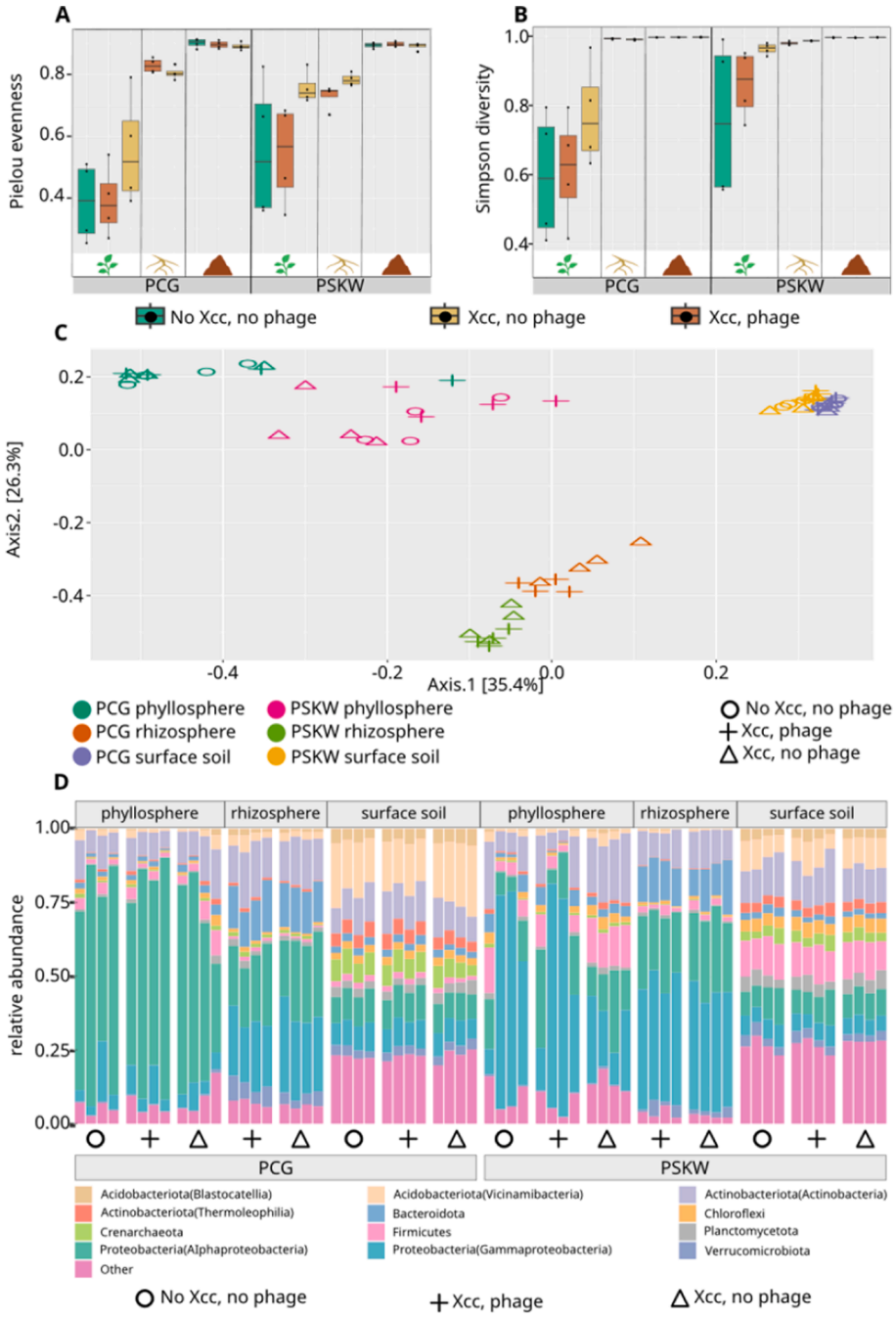


**Fig. 4.** Diversity of the samples from the field experiment. Differences in evenness and richness (A, B) occurred between sample environment: surface soil had both the highest evenness and richness, followed by rhizosphere and phyllosphere samples. No differences in the richness or evenness were observed between the control (◯) Silwet gold (△) and phages with Silwet gold (+). (C,D) Samples originating from different environments had very different microbial compositions. Similarly, the same environments at both sites had slightly different compositions. However, there is no indication that adding phage or Silwet gold has affected these metrics.

understanding of how phage biocontrol can affect the microbiome, whether and when more experiments are required. For instance, other pathogens like *Pseudomonas syringae* and *Streptomyces*, in contrast to *Xanthomonas campestris* pv. *campestris*, might have members that are beneficial for plant health, e.g. by inhibiting fungal growth (Passera et al., 2019; Ashfield-Crook et al., 2018). Indeed, recently some *Streptomyces* phages were shown to unintentionally kill beneficial *Streptomyces* species, making wheat plants more prone to fungal infection (Ashfield-Crook et al. 2018). Although our molecular approach can help to determine if the use of bacteriophages in such cases has undesired off-target effects, conventional microbiological techniques including host range tests and small pot trials with beneficial bacteria closely related to the pathogen, should be included,as these enable to unambiguously test if there might be off-target effects with adverse consequences.

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*CRediT authorship contribution statement*

**Kiandro J. Fortuna:** Formal analysis, Data curation, Writing – original draft, Visualization. **Márton Szoboszlay:** Conceptualization, Validation, Formal analysis, Investigation, Writing – review & editing. **Dominique Holtappels:** Investigation, Writing – review & editing. **Rob**

**Lavigne:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Christoph C. Tebbe:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Jeroen Wagemans:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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