Received: 12 June 2021 | Accepted: 29 March 2022

DOI: 10.1111/2041-210X.13876

Methods in Ecology and Evolution BRITISH ECOLOGICAL SOCIETY

REVIEW

Soil bacterial biodiversity characterization by flow cytometry: The bottleneck of cell extraction from soil

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Handling Editor: Robert B. O'Hara

Abstract

1. The importance of soil biodiversity is increasingly recognized in agriculture and natural resource research and development. Yet, traditional soil biodiversity assessments are costly and time-consuming, limiting the extent and frequency of sampling and analysis in space and time. Flow cytometry (FCM) is a powerful technique to characterize cell communities due to its high robustness and accuracy, requiring only a short time for the characterization. Therefore, FCM could expand soil research capabilities by allowing the characterization of different aspects of bacterial biodiversity. However, this implementation of FCM requires the previous dispersion, separation and purification of bacteria from complex soil matrices. Moreover, soil monitoring programs or evaluation of soil management practices require high-throughput analysis. In this context, soil processing protocols need to consider not only an adequate recovery of undamaged, representative and pure soil bacteria, but also short-time processing requirements. Although soil processing protocols have been reported over time, to our knowledge, there is no recommended soil extraction protocol for high-throughput analysis of bacterial biodiversity by FCM.
2. We reviewed the state-of-art of the use of flow cytometry in scientific research and the protocols used for the extraction of bacteria from soil. We analysed the literature to take stock of the diversity of methodologies for soil processing and applications of flow cytometry in bacterial characterization considering abundance, diversity, community structure and functional properties.
3. This review provides several lines of evidence of the use of flow cytometry for soil bacterial biodiversity (SBB) characterization, highlighting its potential for soil monitoring and studies on soil bacterial community dynamics. The review also highlights and discusses the most relevant constraints and research gaps that need to be considered for high-throughput analysis of SBB by FCM, such as evaluation of scale-down, new reagents for and methods of purification, threshold of bacterial recovery efficiency and selection of a standardized and validated protocol.

4. We proposed a protocol for soil bacterial extraction for high-throughput analysis of SBB by FCM and we provided detailed databases of systematized information that would be useful to the scientific community.

KEYWORDS
flow cytometry, high-throughput analysis, soil bacterial biodiversity, soil bacterial extraction, soil bacterial recovery efficiency

1 | INTRODUCTION

Soil organisms, namely soil biodiversity, are increasingly recognized as pivotal in agriculture and natural resource research and development (e.g. FAO et al., 2020; WWF, 2020). Soil biodiversity supports and regulates soil processes, contributing to ecosystem functioning and services (El Mujtar et al., 2019). Therefore, there is a growing demand for soil biodiversity knowledge world-wide (e.g. FAO et al., 2020). This knowledge requires characterization of species abundances but also of taxonomic and functional diversity (e.g. Naeem et al., 2016). Yet, soil biodiversity assessments are by far less commonly used than physicochemical assessments (Bünemann et al., 2018). This bias is in part due to their intrinsic implementation problems (e.g. time-consuming and expensive methodologies) limiting the extent and frequency of sampling and analysis in space and time.

Metagenomics and amplicon-sequencing (e.g. 16S or ITS target sequencing) are currently the cultivation-independent methods most commonly used to characterize microbial communities. They allow the characterization of relative abundance and taxonomic diversity and community composition of environmental samples (Pérez-Cobas et al., 2020). The data from these technologies are not quantitative and are only predictive of the community functional potential (Douglas et al., 2020; Jansson & Hofmockel, 2018). Both technologies are based on DNA extracted from environmental samples which generally comprises intra- and extra-cellular DNA, and amplicon-sequencing is prone to technical biases (Dopheide et al., 2019; Sun et al., 2020; Wasimuddin et al., 2020). These methods are still too expensive and time-consuming to be applied when fast changes in community composition need to be detected and are not easily optimized to near real-time evaluations (Liu et al., 2018). Therefore, alternative and complementary methods are required to provide knowledge about microbial biodiversity and understanding of microbial diversity dynamics.

Flow cytometry (FCM) is a technology that rapidly analyses single cells suspended in a buffered salt-based solution (McKinnon, 2018). It has been gaining interest in the field of microbial ecology due to its capacity not only to characterize a high number of cells/samples in a short time but also to provide phenotypic diversity measures contributing to microbial dynamic assessments (Heyse et al., 2021; Liu et al., 2019; Pereira et al., 2022). Rapid and easy-to-use tools have been established for nearly fully automatic analysis and visualization of microbial community data based on flow cytometry. An overview of the full computational analysis pipeline of flow cytometry data in microbial ecology has been recently reviewed (Rubbens & Props, 2021).

Cytometric fingerprinting methods for microbial community analyses are broadly classified as based on bins or gates. Bin methods are based on single-step discretization ('binning') of phenotypic parameters (García et al., 2015; Props et al., 2016; Rogers & Holyst, 2009), while gate methods are based on cell clusters defined by boundaries on 2D cytometric histograms (Günther et al., 2012; Koch et al., 2013). Although both methods have advantages and disadvantages (Liu et al., 2018; Rubbens et al., 2021), they are currently commonly used in microbial ecology. They provide fast assessments of microbial diversity dynamics (Props et al., 2016) and stability properties of microbial communities (Liu et al., 2018) and also reveal ecological mechanism modelling microbial community structures (Liu & Müller, 2020). Correlations between microbial abundance and diversity assessments based on FCM and amplicon-sequencing have been reported (Heyse et al., 2021; Props et al., 2016). Recently, a new method for microbial diversity dynamics based on cytometric fingerprinting has been evaluated in synthetic and natural freshwater microbial communities (Rubbens et al., 2021). Recent advances in microbial community analysis from machine learning of FCM data have also been reported (Özel Duygan & van der Meer, 2022). These technological advancements enable automated data acquisition and analysis prompting research on microbial biodiversity (De Vrieze et al., 2021; Haberkorn et al., 2021; Özel Duygan & van der Meer, 2022; Pereira et al., 2022).

Regarding soil microbial biodiversity characterization, the use of FCM requires the previous dispersion, separation and purification of microbes from the soil matrix. This soil processing is essential to fulfil FCM requirements, avoiding interferences or biases associated with aggregated cells or soil particles. The soil matrix has a complex nature and includes the aggregates of different sizes composed of pores and solid material (Wilpiszeski et al., 2019). Aggregate formation depends on several factors (e.g. vegetation, soil organisms, minerals and organic matter interactions) and involves a wide range of extracellular polymeric substances produced by micro-organisms (e.g. Costa et al., 2018). Therefore, the extraction of microbes from the soil matrix could be a process difficult to perform. Monitoring programs and evaluation of soil management practices require short-time processing

methodologies. Consequently, protocols designed for soil microbial extraction should also consider high-throughput procedures. Different protocols for soil processing have been reported (e.g. Lindahl & Bakken, 1995). However, to our knowledge, there is no recommended soil processing protocol for high-throughput characterization of soil bacterial biodiversity (SBB) by flow cytometry. The lack of a reference protocol for soil bacterial extraction (SBE) could be a critical limitation for FCM implementation in soil research.

We reviewed the state-of-art in the use of flow cytometry and the protocols used for extraction of bacteria from soil in order to: (a) provide an overview of FCM implementation for SBB characterization; (b) compare available protocols for SBE, aiming to inform on the appropriate threshold for soil bacterial recovery and, if possible, to identify a standardized protocol for soil processing; and (c) discuss application potentials, constraints and research gaps for the use of FCM for the characterization of SBB.

Details of the two systematic literature searches performed on Scopus database (Search1 and Search2) and the methodology used to cover our research goals are presented in Table S1 and Figure S1. The review was organized in two sections. In the first one, based on Search1, we globally reviewed the use of FCM on scientific research and identified specific research goals of FCM implementation on microbial ecology and particularly in soils. In the second one, based on Search2, we characterized and compared protocols for SBE.

2 | OVERVIEW OF FLOW CYTOMETRY IMPLEMENTATION ON SCIENTIFIC RESEARCH

A total of 12,960 articles (1977–2020) were retrieved from the first systematic literature search (Search1, based on the occurrence of the term '*flow cytometry*' in the article title), revealing a wide use of FCM in scientific research. A subset of 1880 articles (1978–2014), focused on the use of flow cytometry for microbial characterization, was identified by reading the title, abstract and keywords of all articles from Search1. These studies considered environmental samples (e.g. water, sediments, soils), cultures of specific micro-organisms (e.g. bacteria, virus, yeast) or clinical samples (e.g. urine samples) and represented 14.5% of the total, highlighting that FCM has been extensively used for microbial research. The range of years of publication of this subset was similar to that of the total articles revealing that FCM use in microbial research is not recent. Network analysis based on terms from the title and abstract of these articles revealed six clusters (Figure S2), which highlighted some of the research areas of microbiology in which FCM has been applied (e.g. microbial ecology and clinical, industrial and environmental microbiology).

The frequency analysis based on a subset of 37 articles (1982–2019), including the term *soil* in title, abstract or keywords of the articles from Search1 (Table S2), revealed three specific research goals of soil microbiology assessed by FCM: (a) indirect soil characterization based on biosensors (five articles), (b) determination of bacterial abundance (five articles) and (c) evaluation of physiology and activity of bacteria (one article). Spiked bacteria (i.e. bacteria added to soils), such as biosensors and specific target species/strains, were mostly considered among these articles. The rest of the articles required previous cultivation of bacteria from soil or mentioned soil as part of the environmental microbiology characterization.

3 | CHARACTERIZATION AND COMPARISON OF PROTOCOLS FOR SOIL BACTERIAL EXTRACTION

3.1 | Trends on soil bacterial extraction protocols

A total of 109 articles (1967–2019) were retrieved from the second systematic literature search (Search2, see details in Table S1 and Figure S1). The database included 138 protocols (Table S3). More than 90% of the protocols focused on bacteria, an expected result considering the keywords used for this search. The 76% of the protocols focused on native cells (i.e. cells that naturally occur in soils), while the remaining focused on spiked cells (i.e. cells added to soils). However, recovery efficiency (i.e. the percentage of total bacteria recovered from soil) was only reported in the 31% and 51% of the protocols focused on native and spiked cells respectively. Surprisingly, only 58% of the protocols reported complete or partial validation. Globally, frequency analyses revealed high variation for almost all protocol variables considered (Figure 1). In particular, methods used for soil bacterial characterization mostly involved plate count (28%), and optical (14%) and electronic microscopy count (15%), while other methods such as isotopic labelling, metabolic activity or specific metabolite content, and flow cytometry were less reported (Figure 1).

A high number of soil diluents for SBE have been reported, although water has been the most frequently used (Figure 1). Other diluents, such as sodium pyrophosphate, sodium cholate or deoxycholate, phosphate buffers, tris buffer and saline solution, have been also used in order to allow a higher soil dispersion and recovery of bacteria (Figure 1, Table S4). However, globally, around 60% of the diluents were used without comparative evaluations (Figure 2, Table S4). Controversial results were highlighted due to a trade-off between positive effect on soil dispersion and negative effect on soil bacterial physiology, viability and representativeness (Figure 2, Table S4). Diluents have also been used in several diluent/soil ratios, although more than 70% of the protocols used a diluent/soil ratio lower than 10 (Figure 1). This parameter was normally related to the final volume that each methodology of dispersion could handle and was also determined by a trade-off between the amount of soil analysed and the volume of diluent needed for dispersion. This trade-off could affect protocol scale-down (i.e. protocols working with micro-tubes and low amount of soil). Protocols for SBE encompassed three main steps: soil dispersion, bacteria and soil separation, and bacterial purification from debris and soil particles of low size. These steps have been performed using a wide combination of treatments. Moreover, protocols varied from simple protocols, involving

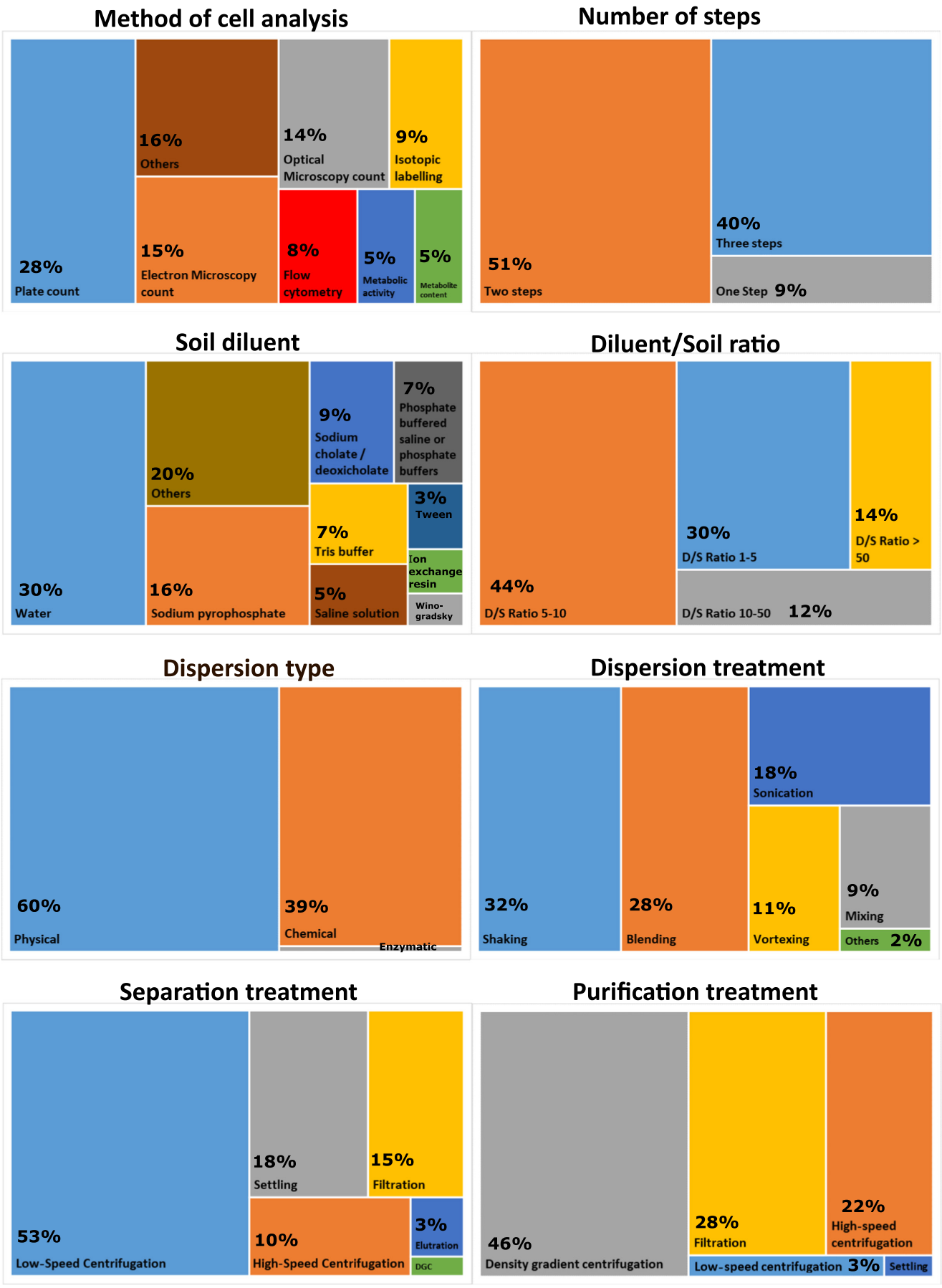

FIGURE 1 Overview of the variation among categorical variables recorded for the 138 protocols analysed

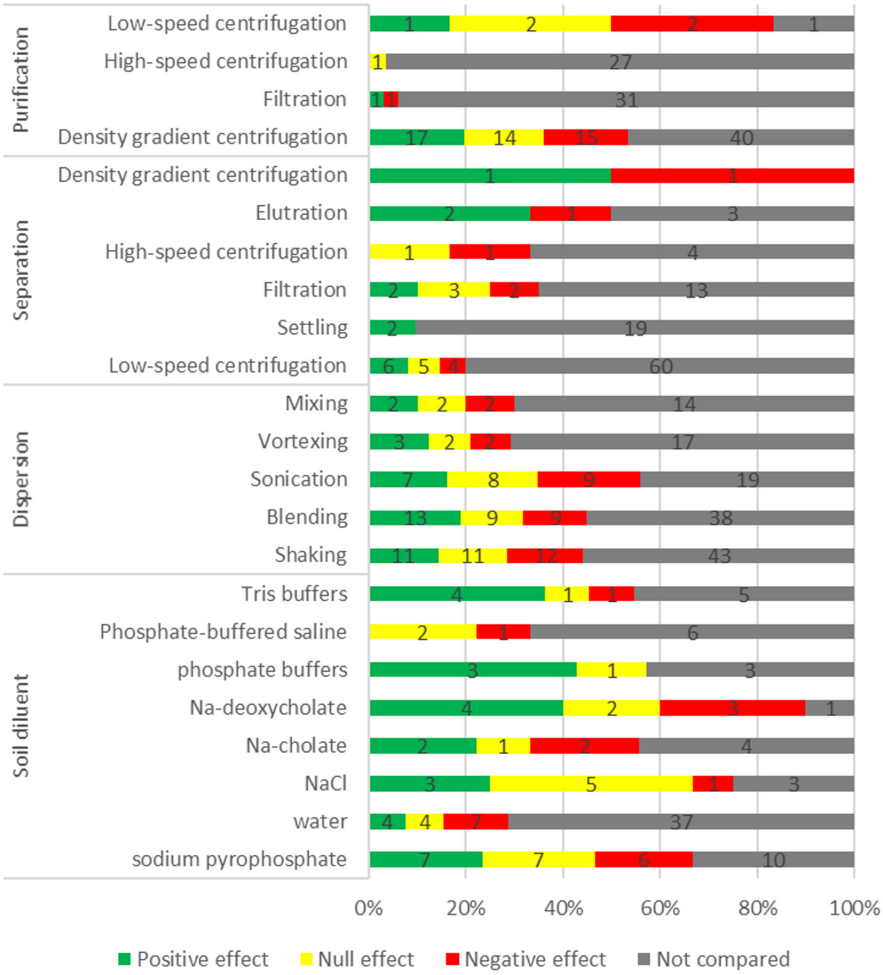

FIGURE 2 Overview of reports of soil diluent and soil treatment effects on soil bacterial extraction (e.g. recovery, purity, viability) among the 138 protocols reviewed. Soil diluents and soil treatments more frequently used (based on frequency analysis, see Figure 1) are presented. Positive, negative or neutral effects of each soil diluent or treatment were based on comparisons with other soil diluents or treatments respectively. See Tables S4 and S5 for details on the reported effects

one single step to more complex protocols, comprising three steps and step repetitions. One-step protocols have been used at lower frequency than the others (Figure 1).

Three types of dispersion methods were identified, namely physical, chemical and enzymatic dispersion (Figure 1). However, chemical and enzymatic types were always combined with physical dispersion and mostly referred to the use of soil diluents different than water to increase soil dispersion (e.g. detergents, chelating compounds, ion exchange resin) and to the addition of enzymes for the degradation of extracellular polymeric substances. Therefore, dispersion treatments were defined by physical dispersion, which covered several options (Figure 1, Table S5). Among physical dispersion methods, shaking and blending were the most frequently used, although sonication, vortexing and mixing were also reported (Figure 1).

Separation and purification treatments also involved several methods, but low-speed centrifugation (LSC) and density gradient centrifugation (DGC) represented around 50% of the methods used respectively (Figure 1, Table S5). Different reagents were used for DGC, such as Nycodenz, Percoll, Sucrose, Metrizamide, Ludox and Ficoll. Nycodenz was the reagent most frequently used (Table S5) due to the high purity (i.e. the ratio between extracted cells and contaminant soil particles) it provides. However, negative impacts of Nycodenz and other DGC reagents on viability (i.e. the integrity of cells) or representativeness of extracted bacteria have been reported (Table S5). Moreover, positive, negative and null effects have been reported for almost every method of dispersion, separation and purification (Tables S4 and S5, Figure 2).

A total of 16 articles reported the use of FCM to characterize SBB. The 18 associated protocols for SBE revealed the following trends: (a) several soil diluents used, (b) combination of dispersion by vortexing, separation by LSC and purification by DGC through Nycodenz, (c) native and/or spiked cells analysed, (d) recovery efficiency reported only for spiked cells and (e) scale-down for microtubes frequently used. These articles revealed that the use of FCM for soil bacterial characterization was first reported in 1991. Since then, FCM has been used alone or in combination with other methods, showing a tendency of higher use in the last three decades (Figure 3).

3.2 | Recovery efficiency of bacteria from soils

Although recovery efficiency was determined by different methods (e.g. microscopy count, plate count, microbial activity), here, we only provide results based on microscopy count. We focus on microscopy count because it is usually considered as a reference method and,

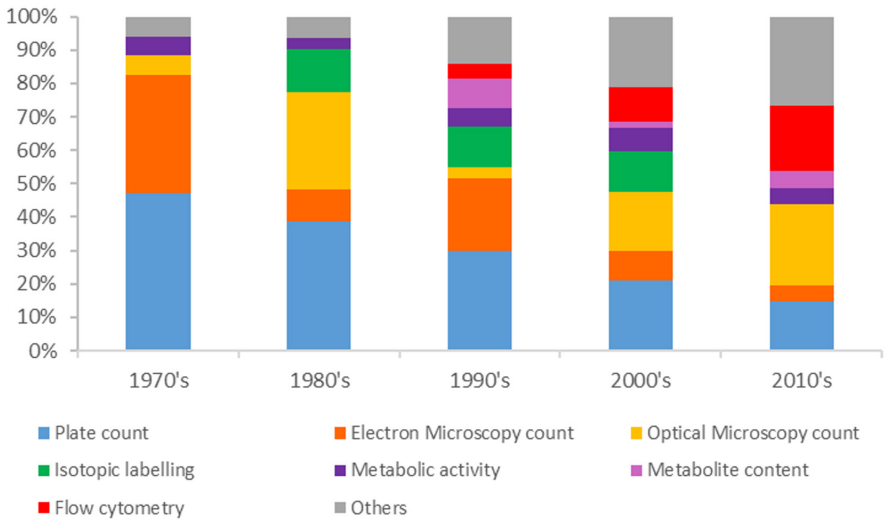

FIGURE 3 Temporal report of the methods used for soil bacterial biodiversity characterization among the 138 protocols reviewed

therefore, more data were available. Recovery efficiency showed high variation among protocols (0.5%–97%, Table S6). This variation was in part explained by the type of cells analysed and the protocol used for SBE (Figure 4). Higher recovery was globally detected for spiked than for native cells (Figure 4). A similar trend was detected on data from articles working simultaneously with both type of cells, although the difference was not significant due to the low sample size (Figure 5). Considering native cells, higher recovery was detected for protocols using only a dispersion step (H = homogenization) compared to those adding LSC or DGC for separation/purification steps (Figure 4). The lowest recovery was observed for protocols adding DGC compared to LSC, alone or in combination with high-speed centrifugation (HSC), or to sedimentation (S) (Figure 4). Recovery efficiency was also affected by soil conservation and use, while differences were globally not detected for soil texture (Figure 5). However, interaction between soil texture and extraction protocol could be expected. In fact, when considering protocols combining H-LSC (the only one with enough data for the analysis), the effect of soil texture was detected (Figure 5). Considering soil use, the difference was significant between agricultural and dune, but sample size was clearly imbalanced for agricultural use which could also include several crop types. Results from soil conservation revealed that drying the soil decreased recovery efficiency. However, we could not rule out an interaction effect with the protocol type as four of the five cases of dry soils corresponded to H-DGC protocols which were also characterized by low recovery efficiency.

Variation in recovery efficiency could be also due to differences in the methodologies used for bacterial counting by microscopy. Protocol conditions and dyes applied in microscopy count could affect unspecific staining of soil particles and, consequently, bacterial count. In general, acridine orange was the dye most frequently used (64%); however, ethidium bromide, SYBR green II, DAPI and aniline blue were also used (Table S6). Moreover, information about staining protocols used for microscopy count was generally not clearly provided in the articles.

Correlation between recovery efficiency and soil physicochemical properties was detected for percentage of clay (rho = −0.73,

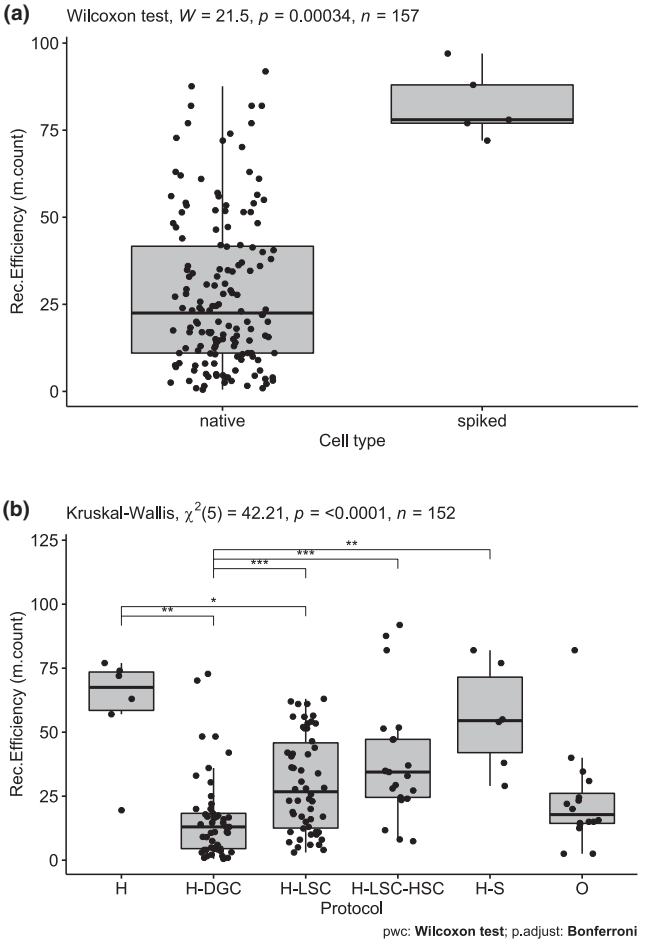

FIGURE 4 Cell (a) and protocol (b) effect on recovery efficiency of soil bacteria. Rec.Efficiency (m.count) represents the percentage of total bacteria recovered from soil samples based on microscopic count analysis. In boxplots, lower and upper box boundaries represent first and third quartiles, respectively, the line inside the box is the median, and lower and upper error lines represent minimum and maximum respectively. Dots represent samples analysed for each group (jitter function was used to visualize all samples). DGC, density gradient centrifugation; H, homogenization; HSC, high-speed centrifugation; LSC, low-speed centrifugation; O, other combinations; S, sedimentation

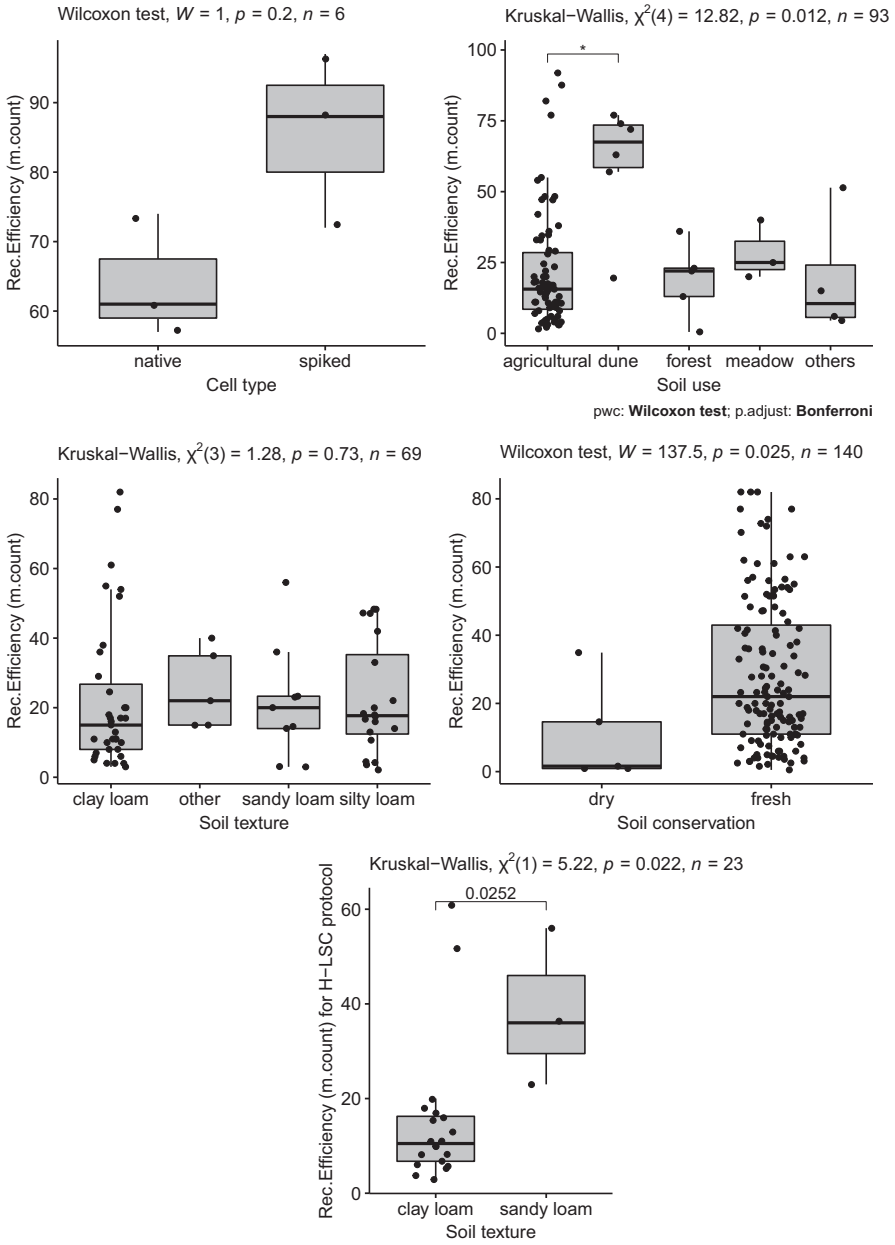

FIGURE 5 Effect of cell type (a), soil use (b), soil texture (c), soil conservation (d) and soil texture for H-LSC protocols (e) on recovery efficiency of bacteria from soils. Rec.Efficiency (m.count) represents the percentage of total bacteria recovered from soil samples based on microscopic count analysis. In boxplots, lower and upper box boundaries represent first and third quartiles, respectively, the line inside the box is the median, and lower and upper error lines represent minimum and maximum respectively. Dots represent samples analysed for each group (jitter function was used to visualize all samples)

p = 8.119e-07, N = 34) and organic matter (rho = 0.50, p = 0.02049, N = 21). Correlation was not detected for pH (N = 65), organic carbon content (N = 28) and total C or N (N = 16 and 13 respectively).

3.3 | Bibliographic structure of soil bacterial extraction protocols

Network analysis, based on references cited for the 138 extraction protocols, revealed that six articles accounted 37% of the total citations (Figure 6). Bakken and Lindahl (1995) was the most cited article, followed by Fægri et al. (1977), Bååth (1992), Lindahl and Bakken (1995), Bakken (1985) and Courtois et al. (2001). Although these articles basically used a homogenization-centrifugation method, they differed on procedure complexity, treatment conditions and soil diluent used (Tables S4 and S5). This analysis also revealed that the protocols used are globally based in a few reference protocols, and that innovation has been related more to modifications on reference protocols (mostly without validation) than in developing or evaluating new methods for dispersion, separation and purification steps.

3.4 | Classification of protocols for soil bacterial extraction

Reinert's method and post hoc correspondence factor analysis based on 106 available abstracts (corpus text) revealed five classes, each of them targeting different research topics (Figure 7). The first two factors explained 61.9% of the variation. This analysis reveals that most of the articles were not focused on the development or evaluation of soil processing protocols, but on the use of soil processing protocols

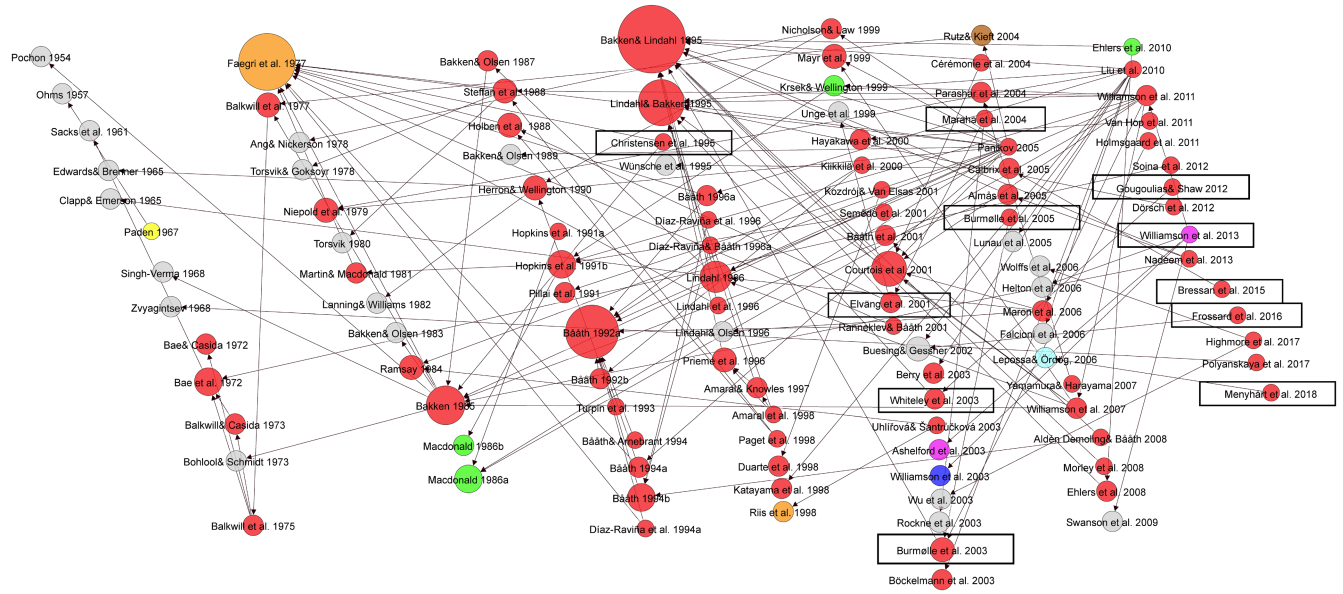

FIGURE 6 Network visualization of bibliographic reference citations from the 138 protocols. Node sizes are scaled according to the number of citations. The direction of the arrows indicates the direction of the citation. Node colours correspond to: red (bacteria), green (micro-organism), orange (bacteria and fungi), violet (bacteria and virus), yellow (virus), brown (bacteria and archaea), blue (fungi) and grey (article cited but not reviewed). Rectangles indicate articles using flow cytometry for soil bacterial characterization

in soil microbial research. This information indicates that protocol validation and standardization is still required.

The classification analysis based on a matrix of six categorical variables (120 protocols) allowed to identify seven protocol types (Figure S3, Table 1). The first two factors explained 53.8% of the variation. The first plane of the correspondence factor analysis revealed a spatial organization in three groups. Factor 1 separated class 5 from the other classes, and factor 2 separated classes 3 and 4 (Figure S4).

Comparing the results of both classification analyses (corpus text and categorical matrix), different combinations of protocols and methods of analysis after cell extraction were detected (Table S7, Figure S5). This result is a consequence of the variation detected in the protocols combining different soil diluent, soil/diluent ratio and treatments. Articles using flow cytometry revealed variation on the combination of the main categorical variables analysed. This result agrees with those from the frequency analysis and indicates variation in the type of protocols used for cell extraction previous to flow cytometry characterization.

4 | DISCUSSION

Our work provides evidence about the growing use of flow cytometry in the study of SBB. It also identifies its main constraints and research gaps, mostly related to the lack of a standardized and validated protocol for SBE. We focused on SBB but results of our work could be probably extrapolated to other soil micro-organisms with the exception of filamentous fungi.

Our literature searches depended on the keywords and on the database used; and, therefore, were not exhaustive. They recovered information until March 2020 and December 2019 (Search1 and Search2 respectively); consequently, articles published after those periods were not included in the database. Some of them (2021–2022) are, however, discussed below.

4.1 | Application of flow cytometry for soil bacterial biodiversity characterization

Flow cytometry use for soil bacterial characterization was first proposed by Page and Burns (1991) as an alternative method to determine bacterial abundance due to the limitations of plate and microscopy counts (e.g. focused on viable bacteria, laborious and time-consuming methods). Thereafter, enumeration of soil bacteria has been one of the main goals of FCM in soil research. Quantitative detection by FCM of native cells extracted from soils has been reported based on the use of ethidium bromide (Christensen et al., 1995), SYBR Green (Bressan et al., 2015; Frossard et al., 2016; Whiteley et al., 2003), propidium iodide (PI) and Syto-9 (Shamir et al., 2009). Reports on native soil bacteria revealed: (a) high repeatable abundance estimates, enough sensitivity to detect treatment and site differences in bacterial abundance and efficacy to assess soil bacterial abundance from relatively small amounts of soil (e.g. Khalili et al., 2019); (b) fairly good correlation between FCM and microscopy count and good potential for high-throughput quantification of bacterial abundance in soils, sediments and sludge (e.g. Frossard et al., 2016); and (c) significant correlation between FCM and molecular methods, and enough sensitivity to detect crop management differences in bacterial abundance (Bressan et al., 2015; Heyse et al., 2021; Khalili et al., 2019).

FIGURE 7 Post hoc correspondence factor analysis performed after Reinert's classification of active forms in the abstracts of 106 articles retrieved from the literature search. The size of each term is proportional to its Chi-square value and indicates the association strength with the class

Reviewed articles also revealed that FCM has been used to determine soil bacterial phenotypic diversity, such as: (a) viability or physiology of native or spiked soil bacteria (Maraha et al., 2004; Shamir et al., 2009); (b) changes in cell volumes and DNA contents of native soil bacteria during culture (Christensen et al., 1995); (c) bacterial quorum sensing (e.g. Burmølle et al., 2005), genotoxins occurrence (Norman et al., 2006) and metal bioavailability in contaminated soils (Hurdebise et al., 2015) using biosensors and biotechnology tools; and (d) genetic diversity of specific soil bacterial groups, using 16S rRNA gene probes combined with fluorescence-activated cell sorting (Gougoulias & Shaw, 2012). However, the use of dyes for bacterial viability or physiology assessment by FCM requires critical evaluation because several factors could affect their performance. Different affinity of dyes between living and dead cells, background fluorescence and bleaching effects are common, and assays should be validated with target micro-organisms (e.g. Davey & Guyot, 2020; Rosenberg et al., 2019). This topic was out of the scale of our review; however, it should be considered for soil microbial ecology, due to the complexity and heterogeneity of the soil matrix and microbial communities.

Flow cytometry allows the estimation of diversity metrics and helps to assess the dynamics of microbial diversity (Liu et al., 2018; Props et al., 2016). Microbial single-cell research of environmental samples could be also performed based on multiparametric approaches, including FCM, allowing co-assessment of the taxonomy, function and metabolism of microbial communities (Pereira

TABLE 1 Summary of categorical variable modalities associated with each class of the classification analysis

Class	Dispersion treatment	Separation treatment	Purification treatment	Soil diluent	Diluent/soil ratio	Extracted cells
1	Blending	Not performed	Density gradient centrifugation	—	—	Native
2	Others	High-speed centrifugation, Not performed	Others	Others	—	Native
3	Vortexing	Low-speed centrifugation	High-speed centrifugation	Others, PBS	1/5	Spiked
4	Others	Settling, filtration	Not performed	Sodium pyrophosphate and Tris buffers	>50	Native/Spiked
5	Mixing	Low-speed centrifugation	Filtration	Water	Several, 10–50	
6	—	Not performed	—	—	5–10	Native/Spiked
7	Sonication and shaking	Others			5–10	Native

Notes: Combination or less frequent methods. Symbol '—' indicates that no specific modality was associated with the class.

et al., 2022). These developments are supported by data analysis pipelines (e.g. Rubbens & Props, 2021), enabling online and also real-time evaluations (Favere et al., 2020; Haberkorn et al., 2021). Among the reviewed articles focused on soil, the analyses of community structures based on cytometric fingerprinting, considering both gate and bin methods, have been reported for microbial photoautotroph (Menyhárt et al., 2018). This report suggests that bacterial biodiversity characterization by FCM could be successfully performed. However, as SBE could affect phenotypic parameters (e.g. viability, activity), FCM is still constrained by the lack of a standardized and validated protocol for SBE as we discussed below.

4.2 | Constraints of flow cytometry use for soil bacterial biodiversity characterization

4.2.1 | Available protocols require improvement and validation

High heterogeneity among the available protocols, low implementation of protocol validation and, consequently, contradictory reports regarding the effects of reagents or treatments on bacteria extracted from soils were found. Globally, protocols have been mainly defined by a trade-off between suitable recovery of cells and cell viability and high-throughput requirements.

Articles using FCM for soil bacterial characterization showed higher variation for dispersion than for separation/purification steps. Requirements of further validation and improvement in protocols have been reported, especially for native cells (Bressan et al., 2015; Frossard et al., 2016). Khalili et al. (2019) optimized a method to quantify soil bacterial abundance by FCM, combining vortexing in sodium pyrophosphate/Tween 80 and DGC through Nycodenz. However, the negative impact of sodium pyrophosphate and Nycodenz on viability or representativeness of extracted bacteria has been reported (Tables S4 and S5). Therefore, this protocol probably should not be recommended for functional and structural diversity characterization. Bressan et al. (2015), on the other side, used a simple protocol combining shaking in saline solution and LSC. This protocol allowed the correct count of bacterial cells in a background of soil particles but it required the previous definition of an optimal gate based on the detection of spiked bacteria. This procedure could be inadequate for other soils and bacterial communities, requiring therefore a gate definition for each case. The evaluation of available cytometric fingerprinting methods could be a good option to overcome this problem. However, the protocol still requires validation for a wide range of soil physicochemical properties (Bressan et al., 2015) and also for viability and physiological analysis. Adjustment of pH saline solution will be probably needed because saline solution without pH adjustment showed lower recovery efficiency and change on community structure compared to water (Table S4). Ouyang et al. (2021) proposed a protocol based on physical blending, Tween 20 treatment and centrifugation with 80% Nycodenz. This protocol had the highest cell viability and yield among the alternatives evaluated; however, blending is time-consuming and the use of Nycodenz or Tween 20 should also be validated. Recently, Lee et al. (2021) comparing culture dependent and independent quantification methods reported that FCM was ineffective in counting soil bacteria due to low purity of bacteria extracted from soils and staining of clay particles by the dyes used. However, the purification step of the protocol used was inadequate according to our review.

4.2.2 | Recovery efficiency of undamaged soil bacteria has a threshold

Recovery efficiency is actually very hard to estimate. No single method exists for absolute microbial count in soil due to its complex nature and, therefore, most of the available methods use a pretreatment of soil (Lee et al., 2021). According to our review, recovery efficiency showed high variation but was in general below 25% for native cells. It was affected by several factors (e.g. type of cells and extraction protocols) and conditioned by a trade-off with cell viability. These results agree with recent publications highlighting that step repetitions (re-extractions) or stronger dispersion conditions could increase recovery but decrease cell viability (Lee et al., 2021;

Ouyang et al., 2021). Our analysis of recovery efficiency was based on microscopy count, as this was the method most frequently used. However, different dyes were used among recovered articles and microscopy protocols were mostly not reported. Therefore, we could not rule out the use of soil pre-treatments before bacterial counts. All these factors could also contribute to the high variation detected for recovery efficiency estimates (Lee et al., 2021). However, even considering these constraints, our review revealed that recovery efficiency of native bacteria from soils is low (i.e. most of the bacteria remain in the soil residue after extraction).

The effect of extracellular polymeric substances (EPSs), produced by micro-organisms, on recovery efficiency has not yet been evaluated. EPSs enhance the aggregation of soil particles (Costa et al., 2018), suggesting that different conditions would be required for exhaustive bacterial extraction from soils varying in quantity and composition of EPSs. Considering the report from Redmile-Gordon et al. (2014), the extraction of EPSs from the soil is accompanied by micro-organism lysis in most of the evaluated protocols, constraining, therefore, its use for SBE. Although the particular extraction protocol of EPSs based on cation exchange resin showed no microbial lysis (Redmile-Gordon et al., 2014), its use for bacterial extraction still requires validation as microbial sorption was reported for this type of resins according to our review.

A low efficiency seems to be the suitable threshold for recovery of undamaged cells reflecting the complexity of bacteria and soil matrix interactions. In fact, 90% and 70% of soil bacteria are associated with soil macro- and micro-aggregates respectively (Wilpiszeski et al., 2019). Bacteria extracted with more conservative protocols will be mostly composed of free and loosely attached bacteria. Although these bacteria could be not representative of all soil bacteria, they will be useful for comparative studies such as those required when monitoring or evaluating soil management practices.

The recovery of strongly attached bacteria should also be considered due to the role of this group, and the associated aggregates, on soil processes and functions (Erktan et al., 2020; Gabbarini et al., 2021; Hemkemeyer et al., 2018). However, the extraction of strongly attached bacteria still requires optimization (Ouyang et al., 2021) and it could be affected by several confounding factors (e.g. different recovery efficiency for soils with different aggregate stability or amount and quality of extracellular polymeric substances). High damage of extracted cells could be expected for free and loosely attached bacteria under harsher conditions required for the extraction of strongly attached bacteria. Sequential extraction of bacteria with different levels of attachment to soil has been proposed as an alternative to separately recover both cell groups (Almås et al., 2005; Nadeem et al., 2013; Ouyang et al., 2021). This alternative seems to be a good option for experiments aiming to characterize the biodiversity associated with different soil compartments and aggregates. However, it is time-consuming and therefore less adequate for monitoring soil management practices, particularly when the evaluation of recovery efficiency, viability and metabolic activity of cells are important issues.

Recovery efficiency of bacterial extraction protocols used in combination with FCM has been reported based on spiked cells, as bacterial abundance in original soils could not be determined directly by FCM. However, recovery efficiency evaluation needs to be carried out by a standardized method such as microscopy count, because recovery efficiency of spiked cells (i.e. cells not attached to soil aggregates) was always higher than that of native cells. Comparative studies among available counting methods are highly recommended in order to define a reference protocol for further research. Alternatively, the development of new methods accounting for absolute soil microbial count should be considered.

4.3 | Research gaps on flow cytometry use for soil bacterial biodiversity characterization

4.3.1 | Evaluation of density gradient centrifugation and alternative purification methods

Nycodenz provides extracted cells with high purity, but negative effects on cells were also reported. Therefore, the development/test of new reagents for DGC or robust evaluation of the impact of DGC reagents on extracted cells will be required.

Regarding low recovery efficiency of DGC, the development/test of other purification methods should be explored. In this sense, filtration methods through glass wool, such as those implemented in thymidine/leucine incorporation analysis, could be a suitable option. This method has been robust for activity evaluations.

4.3.2 | Scale-down evaluation

Scale-down of procedures to work with micro-tubes and low amounts of soil samples was scarce, but it was particularly frequent in studies using flow cytometry (e.g. Khalili et al., 2019; Maraha et al., 2004; Whiteley et al., 2003). This is a promising aspect as it allows the processing of high number of samples and could make protocols more accessible for a high number of laboratories (e.g. micro-centrifuges are more common than centrifuges for large sample volumes), contributing to protocol standardization. The possibility to manipulate and process small samples allows compositional (/functional) analysis of micro-environments (i.e. different rhizospheric subregions), thus approaching variations and biases across better defined positional scales in soil niches of interest. New protocols for SBE should consider and/or validate the performance on scale-down conditions. Filtration through glass wool has been useful to harvest intact cells; however, scale-down of glass wool filtration is not yet available. The combined use with spin columns could be an option for scale-down.

4.3.3 | Proposal of a protocol for soil bacterial extraction useful for flow cytometry

Our review revealed that a standardized and validated protocol for SBE is still required. Protocol selection should be finally based

on the trade-off among purity, viability and recovery efficiency of extracted bacteria. Reviewed information indicates that free and loosely attached bacteria should be targeted, as these bacteria could be more easily extracted, saving cell integrity and representativeness of community members.

Identified trends and recovered information allowed us to propose a protocol for further validation. A good option seems to be the use of saline solution as soil diluent, vortexing for dispersion, LSC for separation and DGC or filtration for purification.

Saline solution (0.8% w/v, pH 7.5) has lower recovery than strong dispersal agents, but also lower or not impact on extracted cells. It allows to overcome some problems of water, providing colourless extractions, low amounts of impurities, good levels of dispersion and enough recovery and similar community structure compared to the original soil.

For dispersion, among mixing, shaking and vortexing, selection would be based on applicability considering equipment cost and standardization. In this sense, several shaker types (e.g. gyrotory, reciprocal, wrist-action shakers) and shaking conditions (1 min to several hours) have been reported, suggesting that standardization would be difficult. Lower variation was detected for mixing or vortexing. Vortexing has been used in several laboratories, it normally requires only a few minutes, it is well-suited to work with microtubes and can be adapted to process several samples simultaneously. Moreover, vortex equipment is generally available on research laboratories and is not expensive. Standardization of treatment duration and speed would be required, but vortexing seems to be the best option for dispersion.

Low-speed centrifugation has been the method most frequently used for separation, providing good separation between dispersed cells and biggest soil particles. Additionally, it has good performance for scale-down and normally requires less than 30 min. Although a wide range of conditions (e.g. centrifugation speed) has been reported, according to Lee et al. (2021), centrifugation at 1400 *g* seems to be a good option.

Purification is one of the most problematic steps as it has a strong impact on the trade-off between purity and recovery of extracted cells. Density gradient centrifugation provides good purity, it is easy to use and can be scaled down. However, this method still requires the evaluation of its impact on cellular activity, viability and representativeness of the extracted cells. Filtration through glass wool in combination with spin columns could be a good option to replace DGC, but it still requires validation.

5 | CONCLUSIONS

According to our literature analysis, the lack of a standardized and validated protocol for SBE is the major constraint for the use of flow cytometry on soil bacterial quantification and bacterial diversity analyses. We proposed a protocol for SBE to be validated on natural and managed ecosystems and provided detailed databases of systematized information that would be useful to the scientific community. Validation of this protocol would overcome the main bottleneck of SBB characterization by flow cytometry.

ACKNOWLEDGEMENTS

This research was supported by the project PICT-2018-03880 of the 'Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación' and by the postdoctoral fellowship INTA-CONICET of V.A.E.M.

AUTHORS' CONTRIBUTIONS

V.A.E.M. conceptualized and conducted the review, performed the analyses and wrote the first draft of the paper; F.C., L.W., A.L. and P.T. provided inputs and helped in the editing process of the manuscript. All authors were involved in the revision process.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/2041-210X.13876.

DATA AVAILABILITY STATEMENT

Data used in this manuscript have already been published or are openly available in the files Supplementary information, Supplementary tables (Tables S1–S7) and Supplementary figures (Figures S1–S5) located in the INTA repository (http://hdl.handle.net/20.500.12123/11585).

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How to cite this article: El Mujtar, V. A., Chirdo, F., Lagares, A., Wall, L. & Tittonell, P. (2022). Soil bacterial biodiversity characterization by flow cytometry: The bottleneck of cell extraction from soil. *Methods in Ecology and Evolution*, 13, 1388–1401. https://doi.org/10.1111/2041-210X.13876