**Polish Journal of Agronomy**
**2023, 52, 14–21**

doi: 10.26114/pja.iung.503.2023.52.02

# Factors determining the occurrence and number of bacteria of the genus *Azotobacter* in the soil environment

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**Abstract.** Bacteria belonging to the genus *Azotobacter* are microorganisms commonly found in various soils all over the world and capable of fixing atmospheric nitrogen. The biological nitrogen fixation (BNF) process annually supplies approximately 140–170 million tons of this element to the nitrogen cycle, which is of great importance from an ecological and practical point of view. Although the efficiency of atmospheric nitrogen fixation by *Azotobacter* spp. is not high and amounts to 20 mg N per 1 g of glucose used, these bacteria are sensitive to various environmental factors, including soil reaction, contents of organic matter, soil humidity or nutrient content, and their abundance in soils is small. These bacteria secrete numerous biologically active substances into the soil environment, which have a beneficial effect on the development of plants, which from the ecological point of view plays an important role in the functioning of agricultural ecosystems. An additional advantage of bacteria belonging to the genus *Azotobacter*, which speaks for their use in agriculture, is the ability to produce antifungal compounds and solubilization of insoluble phosphates. Research on *Azotobacter* spp. proves that the concentration of hydrogen ions (pH) is a significant factor which determines the presence of this group of bacteria in the soil environment. Many other soil properties have a large impact on the presence and development of this important agricultural group of bacteria.

The aim of the work was to systematize the knowledge on the known occurrence conditions and ecological relationships and interactions between environmental factors and the presence and abundance of *Azotobacter* bacteria in soils.

**Keywords:** soil properties, pH, *Azotobacter* spp., atmospheric nitrogen fixation

## INTRODUCTION

Many species of microorganisms inhabit the soil. Fertile soil can contain up to one billion bacteria per 1 g of fresh soil mass (Gałązka et al., 2016). The composition of microorganisms significantly affects the rate of organic matter decomposition and nutrient cycling, as well as their availability in the soil environment. Soil microorganisms play an essential role in, among other things, the mineralization of organic matter, the formation of soil humus, the supply of nutrients to plants and the reduction of pathogens, thereby contributing to the appearance of soil fertility and wholesomeness (Bielińska et al., 2013; Gałązka, 2019).

Soil is a habitat for the life and multiplication of bacteria of the genus *Azotobacter*. Interest in this group of bacteria is primarily related to their properties that can be applied in agriculture – the ability to fix atmospheric nitrogen and provide it in a bioavailable form to plants, producing several compounds that stimulate plant growth and development (Aasfar et al., 2021; Jain et al., 2021). In addition, bacteria belonging to the genus *Azotobacter* are excellent indicators of soil fertility (Lenart, Chmiel, 2008; Natywa et al., 2013). The abundance of *Azotobacter* spp. in temperate zone soils is low, ranging from a few to several thousand cells in 1 gram of soil. The bacteria are detected in 30–80% of analyzed soil samples collected from various regions worldwide (Kennedy et al., 2004). The occurrence and population size of this group of bacteria is influenced by many environmental factors, i.e. soil properties (pH, organic matter content, moisture content, fertility, C/N ratio) or climatic conditions (Tejera et al., 2005). The abundance of *Azotobacter* spp. varies depending on the depth of the soil profile. These bacteria colonize rhizosphere soil in more significant numbers, and the type of crop grown affects their abundance (Kaviyarasan et al., 2020). Fluctuations in *Azotobacter* abundance reflect changes in the soil environment, as these microorganisms respond strongly to physical and chemical factors (Lenart, Chmiel, 2008; Lenart, 2012; Kozieł et al., 2018). Increasingly, strains of *Azotobacter* spp. isolated from soils are being used to produce biopreparations applicable to agriculture. Therefore, it is essential to know and carefully study the interactions

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between environmental factors and the presence and quantity of this group of bacteria due to the possibility of using the abundance of *Azotobacter* spp. as a parameter for monitoring the biological properties of soils.

This work presents the state-of-the-art on the influence of soil properties on the occurrence and abundance of bacteria belonging to the genus *Azotobacter*.

## OCCURRENCE AND ABUNDANCE OF *AZOTOBACTER* SPP. IN THE SOIL ENVIRONMENT

*Azotobacter* spp. infest many environments such as soil, water, sewage sludge, root and leaf surfaces. These bacteria are found in various climatic zones, with many species appearing in tropical and polar regions (Jensen, Petersen, 1995; Aquilanti et al., 2004b). *Azotobacter chroococcum* and *Azotobacter vinelandii* occur more frequently and in more significant numbers in tropical soils (Aasfar et al., 2021). Literature data show that *Azotobacter* spp. also occurs in rhizosphere soil, confirming that these bacteria prefer fertile environments, which is undoubtedly the plant rhizosphere (Brenner et al., 2005; Sivasakthi et al., 2017).

Concerning the occurrence of the bacteria in question in Polish soils, noteworthy is the pioneering research of Prof. Jadwiga Ziemięcka, who, as early as 1923, published the results of her analyses conducted in 1917–1918 on the presence and abundance of *Azotobacter* spp. in samples of 28 soils from the area of the former Kingdom of Poland. The results indicated that the bacteria existed in about 50% of our country's soils (Ziemięcka, 1923). More recent studies make it worth citing the results of Martyniuk and Martyniuk (2003). These authors, referring to the work of Prof. Ziemięcka, sampled 31 soils from different regions of Poland in 2000. Their study showed the presence of *Azotobacter* bacteria in 52% of the soils, and the abundance of these bacteria ranged from a few cells to almost 10,000 cells in 1 gram of soil. In a study conducted by Zawiślak (1973), the abundance of this group of bacteria in agriculturally cultivated soils ranged from a few hundred to a thousand cells in 1 gram of soil. Lenart (2012) studied many soil samples from the southeastern Poland region and found bacteria belonging to the genus *Azotobacter* in 43% of the soils analyzed. The abundance of this group of bacteria ranged from 1 to 112 cells per gram of soil. According to another study by this author, one of the soils was colonized by about 960 cfu of *Azotobacter* spp. per gram of soil (Lenart-Boroń et al., 2014). Kozieł et al. (2021) determined the abundance of nitrogen-fixing bacteria of the *Azotobacter* genus in 182 soil samples collected from different regions of Poland. These bacteria were present in 67 (37%) of the analyzed soil samples. The total abundance of the studied group of bacteria ranged from 3.2 to 10801 cfu $g^{-1}$ d.m. of soil. Foreign studies obtain similar amounts of *Azotobacter* spp. in soils. It is worth quoting the results of analyses by Becking (1961), who studied the occurrence of *Azotobacter* spp. in numerous soil samples from Europe (155 soils), Africa (101 soils), tropical Asia (43 soils), Australia (30 soils) and South America (52 soils), and showed that the proportion of soils colonized by the bacteria in question on each continent was, respectively: 48%, 22%, 37%, 17% and 19%. In a study by Barnes et al. (2007) involving 256 soil samples from southeastern England, the presence of *Azotobacter* spp. was found in about 48% of the soils tested. In the available literature, very high abundances of the bacterial group in question were shown only in Arizona (USA) soil studies. In some garden soils, more than 500,000 cells were detected in 1 gram (Fuller, Hanks, 1982). Such significant discrepancies in the abundance of populations of bacteria of the genus *Azotobacter* may be due to different soil sampling sites, differences in agronomic practices, plant protection products and fertilizers used, and differences in the properties of the soils studied.

## ENVIRONMENTAL FACTORS AFFECTING THE OCCURRENCE AND ABUNDANCE OF BACTERIA OF THE GENUS *AZOTOBACTER*

Bacteria of the genus *Azotobacter* require an adequate supply of nutrients and energy in addition to good aerobic conditions. In addition, the occurrence, multiplication and abundance of this group of bacteria in soils are linked to their sensitivity to several environmental factors, such as soil pH, soil silt and clay fractions, moisture content or organic matter content (Figure 1).

### Soil pH

Bacteria belonging to the genus *Azotobacter* prefer soils with neutral and slightly alkaline reaction (Sartaj et al., 2013; Gothandapani et al., 2017). In acidic soils ($pH < 6$), these bacteria occur rarely and in low numbers, which is related to lower availability of assimilable nutrients, unfavorable air and water conditions and the presence of toxic aluminum ions ($Al^{3+}$) in the soil solution (Martyniuk, 2008; Mazinani, Asgharzadeh, 2014; Andjelković et al., 2018). The concentration of toxic aluminum compounds in the soil depends on the bedrock and the kind and type of soil (Ochmański, Barabasz, 2000). The most significant impact on agriculture comes from active forms of aluminum (mobile, exchangeable aluminum), which are released from mineral structures as a result of lowering the soil pH below 4.2. In Polish soils, the content of active aluminum forms reaches 100 mg $kg^{-1}$ of soil. However, the harmful effects of aluminum have already been recorded well below this value. Increased concentrations of $Al^{3+}$ ions and an increase in the activity of aluminum fractions in the soil environment are associated with an increase in soil acidification. The harmfulness of aluminum ions is based on the chemical

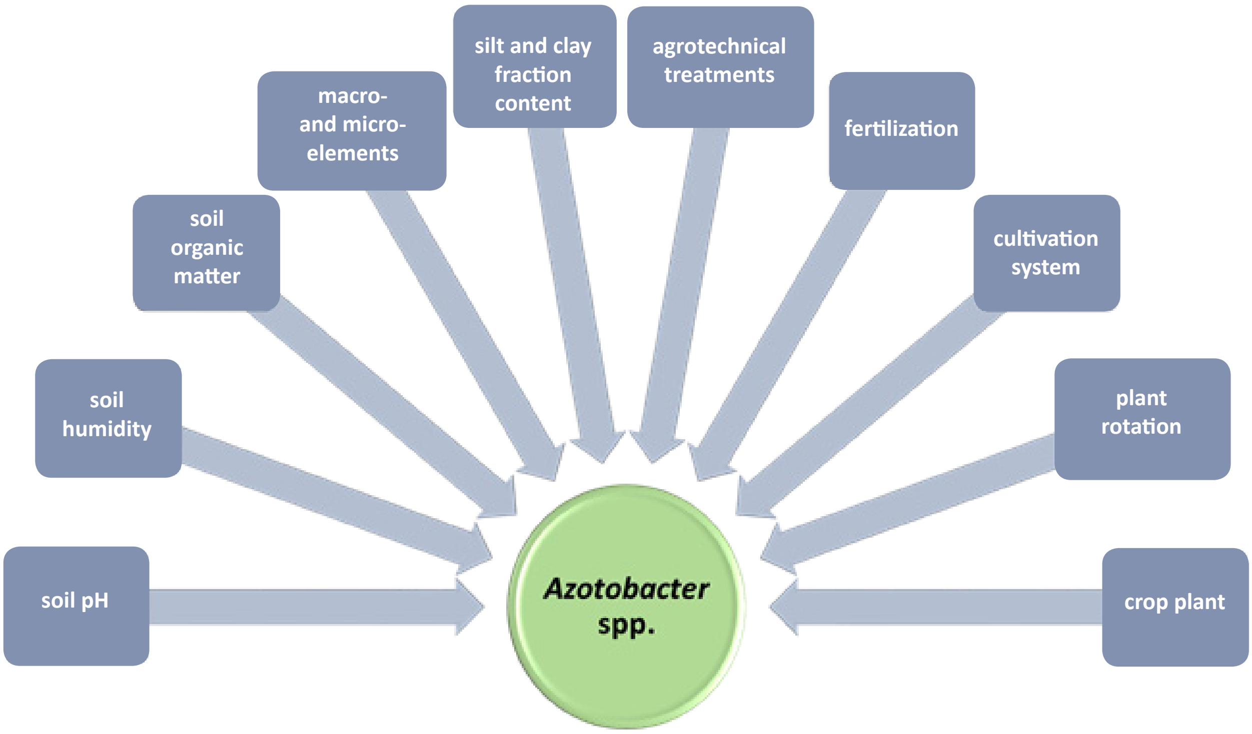


Figure 1. Factors affect occurrence and abundance of *Azotobacter* spp. in soil.

sorption of phosphate and the antagonism of $Al^{3+}$ to $Mg^{2+}$ and $Ca^{2+}$. The increase in soil acidity is caused by the decomposition of organic matter, acid rain, fertilizer use and industrial development, among other factors. Soil acidity is also increased by the leaching of base cations from the soil, which are exchanged by the soil sorption complex for hydrogen and aluminum ions (Zuziak, Jakubowska, 2016). Of the known species, *Azotobacter beijerinckii* is most commonly detected in acidic soils (Aasfar et al., 2021). Also, the environment of alkaline soils is much less conducive to the growth and multiplication of *Azotobacter* spp. than that of neutral soils. In alkaline soils (pH > 7.2), *Azotobacter* abundance ranges from 102 to 104 cfu in 1 g of soil (Becking, 2006). The unfavourable conditions for developing this group of bacteria are caused, among other things, by the limited availability of P and Mg bioavailable forms. Numerous studies by foreign and Polish scientists support the thesis that *Azotobacter* spp. are most abundant in soils with neutral pH (Limmer, Drake, 1996; Aquilanti et al., 2004a; Lenart, 2012; Mazinani, Asgharzadeh, 2014; Ben Mahmud, Ferjani, 2018). The sensitivity of *Azotobacter* genus bacteria to the pH of the soil environment is a species-specific trait. *Azotobacter chroococcum* is able to survive and grow even at pH higher than 9. On the other hand, *Azotobacter salinestris* is sensitive to alkaline soil reaction and its growth is inhibited at pH above 9 (Aasfar et al., 2021). Ramadhan and Issa (2022) analyzed the occurrence and abundance of *Azotobacter* in the alkaline soils of the Zakho region in northern Iraq. The pH of the studied soils ranged from 7.72 to 9.41. The authors observed a lack of growth of the studied genus of bacteria in soils with a pH above 9. They confirmed the direct effect of soil pH on the population of *Azotobacter* spp. in the soil environment. They also found that an alkaline soil reaction is an excellent inducer of atmospheric nitrogen fixation by these bacteria. Mazinani et al. (2012) detected the most abundant populations of *Azotobacter* spp. in soil samples with pH ranging from 7 to 7.4. The numbers of these bacteria decreased at pH above 8 and below 4. Koziel et al. (2021) found that the optimal pH for *Azotobacter* genus bacteria ranged from 6.6 to 7.8, consistent with similar studies obtained by other researchers (Limmer, Drake, 1996; Aquilanti et al., 2004a; Lenart, 2012). Several percent of the analyzed soil samples with a lower pH contained bacteria of the genus *Azotobacter*, while in soils with a pH below 5 no presence was recorded. According to literature data (Ziemięcka, 1923; Martyniuk, 2008), the most abundant populations of *Azotobacter* spp. inhabit fertile soils with a high content of soil fraction <0.02 mm and a pH close to neutral.

Despite the immense influence of soil reaction on the occurrence of *Azotobacter* spp. in various soils, quantitative analyses of soil populations of the bacteria in question often failed to obtain significant correlations between soil reaction and the abundance of *Azotobacter* bacteria. Lenart-Boroń et al. (2014) observed no significant correlations between the quantity of *Azotobacter* spp. in samples of 40 soils collected from agricultural fields and industrialized areas and the reaction (pH in water) of these soils. In a study by Martyniuk and Martyniuk (2003) covering 32 cultivated soils, a significant correlation coefficient between the traits in question was obtained only after one soil colonized by multiple populations of these bacteria was not included in the calculations. Lenart (2012) also noticed a similar phenomenon in her analysis of the abundance of *Azotobacter* genus bacteria in 100 soil samples of different pH, but in this study, the optimum soil pH for the development of the bacteria in the discussion was shifted more in the alkaline direction (pH = 8.2). Very similar results were achieved in a study by Ben Mahmud and Ferjani (2018) comprising 15 soils, in which the highest counts of *Azotobacter* spp. were found in soils with neutral pH.

**Organic matter content**

Soil fertility is an essential factor affecting the colonization of soils by free-living atmospheric nitrogen-fixing bacteria of the genus *Azotobacter*. This is reflected in the study results, indicating significant relationships between soil fertility indices and the occurrence of the discussed group of bacteria in soils. The stimulatory effect of humic and fulvic acids on the growth and nitrogen-fixing efficiency of *Azotobacter* spp. was described by Gaur and Mathur (1966) and Bhardwaj and Gaur (1970). Safari Sinegani and Sharifi (2004) showed that the occurrence of *Azotobacter* spp. in the soils of northwestern Iran is positively correlated with soil organic matter (SOM), organic carbon and total nitrogen content. Studies by Hajibolan et al. (2004) and Mazinani et al. (2012) confirmed the strong relationship between soil organic carbon content and *Azotobacter* spp. abundance. The results of a study by Ebrahimi et al. (2017) on various agriculturally used soils showed a positive, but not statistically significant, relationship between the presence of *Azotobacter* spp. and organic carbon content. A study by Barnes et al. (2007) revealed significant correlation coefficients between humus content and *Azotobacter* spp. abundance in soils near Rothamsted. In Poland, Martyniuk and Martyniuk (2003) showed substantial relationships between the content of organic carbon and total nitrogen and the quantity of this group of bacteria in soils. This dependence is also confirmed by the results of Lenart (2012).

**Contents of macro- and micronutrients**

As the literature shows, *Azotobacter* spp. requires the presence of macronutrients and micronutrients for its development, in addition to adequate soil pH (Balandreau, 1986; Van Niel, 1935; Tchan, New, 1984). Iron and molybdenum are vital because they are part of the active centers of nitrogenase, an enzyme critical in fixing atmospheric nitrogen. In addition, *Azotobacter vinelandii* can produce as many as three types of nitrogenase, depending on environmental conditions:

– nitrogenase I, which contains the coenzyme Fe-Mo-Co (it is made when molybdenum ions are present in the environment),
– nitrogenase II, which has Fe-V-Co as a cofactor (it is produced under molybdenum-deficient conditions, where a vanadium molecule replaces this element),
– nitrogenase III, in which only iron ions are the cofactor (Baj, Markiewicz, 2007; Trncik et al., 2022). Van Niel (1935) demonstrated that the absence of *Azotobacter* spp. in some sandy soils with a pH favourable (pH around 7.2) for the growth of the bacteria in question was due to the shallow content of bioavailable forms of molybdenum in these soils.

In the lab, bacteria belonging to the genus *Azotobacter* thrive best in cultures with neutral pH, requiring relatively high amounts of assimilable phosphorus and magnesium for their development (Jensen, 1965). Thus, it seems that the poorer conditions for *Azotobacter* spp. growth in alkaline soils may be tied to the lower availability of P and Mg bioavailable forms (Sapek, 2014; Mocek, 2015). This is corroborated by studies conducted by Safari Sinegani and Sharifi (2004), who reported a significant correlation coefficient between the population size of *Azotobacter* spp. in soils of northwestern Iran and the content of available forms of phosphorus and potassium.

**Soil type and species**

In the literature on free-living nitrogen assimilators of the genus *Azotobacter*, some papers examined the relationship between the occurrence and abundance of these bacteria and the type and species of soils. Kozieł et al. (2021) recorded the most abundant populations of *Azotobacter* spp. in brown alluvial soils, typical black earth, degraded black earth, and lessive soils. Their most minor numbers were recorded in rusty soils, while no presence of these microorganisms was found in the typical alluvial and podzolic soils. Lenart (2012) found the presence of *Azotobacter* spp. in alluvial soils and cambisols. Siebielec et al. (2015) determined the abundance of nitrogen-fixing bacteria of the genus *Azotobacter* in different soil types under perennial cereal crops. Their highest counts were in eutrophic brown soil and brown rendzina, black earth, and brown alluvial soils. However, the authors of the above-cited works did not show a significant relationship between soil type and the occurrence of *Azotobacter* spp. but their presence was often recorded in fertile soils, e.g., rendzina, alluvial soils and black earth. Rendzinas, as soils formed from various types of calcareous rocks (Smreczak et al., 2018), usually have a reaction close to neutral or slightly alkaline, are rich in calcium and magnesium and can contain up to 6% humus, which is conducive to the occurrence in them not only of *Azotobacter* spp. but also of other groups of bacteria (Becking, 1961; Limmer, Drake, 1996; Grządziel et al., 2019). Likewise, most of the alluvial soils are classified as fertile and productive soils, among other things, because they tend to be humus-rich, contain abundant silt-clay fractions that largely determine the sorption complex, and generally have an alkaline or neutral reaction (Skłodowski, Bielska, 2009; Mocek, 2015), i.e. have properties that favour the development of free-living $N_2$ assimilators in the soil (Roper, Smith, 1991; Weyman-Kaczmarkowa, Pędziwilk, 1999; Gupta, Roper, 2010; Siebielec et al., 2015). Similar is the environment of black earths, which are also fertile soils rich in alkaline elements (mainly calcium) that favour the accumulation of organic matter and thus the occurrence of *Azotobacter* spp. (Łabaz, Kabała, 2014).

The soil content of the silt-clay fraction with particles <0.02 mm is one of the essential criteria for dividing soils into granulometric groups (soil species). The granulometric composition of mineral soils significantly impacts their physical and chemical properties. Soils containing more silt-clay fractions tend to be more fertile, have a more favourable soil crumb structure and higher water-sorption capacity, are richer in humic substances and have a better reaction than soils poor in these fractions, such as light sandy soils (Ryzak et al., 2009; Skłodowski, Bielska, 2009; Mocek, 2015). The abovementioned factors also positively influence soils' biological life, including the development of microorganisms (Weyman-Kaczmarkowa, Pędziwilk, 1999; Torsvik, Øvreås, 2002; Czaban et al., 2010; Siebielec et al., 2015). Martyniuk and Martyniuk (2003) recorded the highest numbers of bacteria of the genus *Azotobacter* in fertile soils with a high content of silt and clay fractions. This is also confirmed by a study led by Kozieł et al. (2021), who found that silt loam, clay loam, very fine sand and sandy clay were beneficial to the growth of *Azotobacter* spp. as these soil species had the highest abundances of the studied group of bacteria. The least abundant populations of free-living nitrogen assimilators belonging to the genus *Azotobacter* were recorded for loose sand.

## Other factors affecting the presence of *Azotobacter* spp.

In addition to the abovementioned factors that significantly impact the occurrence and abundance of *Azotobacter* spp. in the soil environment, agrotechnical treatments, fertilization, the type of crops grown, or the biological properties of the soil are of great importance.

In a study conducted by Zawiślak (1973), the abundance of *Azotobacter* spp. was determined in soils taken from hillsides in the former Olsztyńskie voivodeship (13 hills permanently sodded and 22 under field cultivation). The author of the study found that *Azotobacter* spp. occurred more frequently and in greater numbers (several hundred to a thousand or more cells per g of soil) in agriculturally cultivated soils than sodded soils. It was likely due to the higher quality of arable soils on slopes as a result of their more careful fertilization, more intensive aeration and crop rotation. This demonstrates that agrotechnical treatments carried out on soils can create conditions more favourable for the development and survival of this group of bacteria. Furthermore, the more abundant occurrence of *Azotobacter* bacteria in soils from zones located higher on slopes was determined by the more favourable soil reaction and higher phosphorus and calcium levels for these bacteria. Soils collected from the lower slopes were characterized by a low content of silt and clay fractions, were poorer in phosphorus and were more acidic. Natywa et al. (2013) evaluated the effect of nitrogen fertilization and a irrigartion treatment on the dynamics of *Azotobacter* under corn. They found a significant effect of varying nitrogen fertilization and irrigation on the abundance of the bacteria group. Nitrogen doses exceeding 80 kg $ha^{-1}$ caused a decrease in the number of bacteria of the genus *Azotobacter*, which was associated with the accumulation of toxic substances, such as ammonia, lowering the soil pH and limiting the development of some groups of microorganisms. Excessive doses of nitrogen fertilizers modify the qualitative composition of biocenoses – the number of *Arthrobacter, Azotobacter*, and *Streptomyces* bacteria is reduced, and imperfect fungi take over the dominance in microbiocenoses (Smyk et al., 1989). On the other hand, the irrigation treatment had a positive effect on the populations of the bacterial group in question, which was most likely associated with an improvement in the abundance of assimilable nutrients in the soil. Safari Sinegani and Sharifi (2004) obtained similar results, finding higher numbers of *Azotobacter* bacteria in irrigated plots. Martyniuk et al. (2007) tested whether the soil tillage system affects *Azotobacter* spp. populations. The results showed no $N_2$ assimilators of the genus *Azotobacter* were detected in the soil under winter wheat grown in the experimental conventional system. In contrast, the abundance of this group of bacteria in the soil under plants grown in the organic system amounted to 120 cells per gram of soil. The results obtained can be justified, among other things, by the more excellent supply of organic matter and more favourable soil pH in the organic system (pH = 6.6) compared to the conventional one (pH = 5.6). The study's authors also noted the importance of liming acidic soils, which ensures their proper functioning and increases fertility by stimulating microbial activity, including the development of *Azotobacter* spp. bacteria. Similar results were obtained by Martyniuk and Oroń (2007), who detected these bacteria in limed light clay with a pH of 7.1. Fertilizing the soil with manure or slurry also creates favourable conditions for the growth of *Azotobacter* bacteria (Starzyk et al., 2013). In addition to the factors mentioned above, the type of crop grown can affect the abundance of this group of bacteria in soils. For example, Strzelczyk (1958) reported that radish and legumes stimulate the proliferation of *Azotobacter* spp. in their rhizosphere. Martyniuk and Martyniuk (2003) observed in their study that bacteria belonging to the genus *Azotobacter* were most abundant (9900 cfu $g^{-1}$ of soil) in soil sampled from red clover crops. Cvijanović et al. (2011) recorded the highest number of these bacteria in soil under soybean crops at full flowering. Kizilkaya (2009) studied the correlation between the frequency of *Azotobacter* bacteria and the biological properties of the soil. The results showed a significant correlation between the abundance of *Azotobacter* spp. and soil microbial biomass, dehydrogenase, beta-glucosidase, alkaline phosphatase and arylsulphatase activity. However, no significant associations were observed between the presence of these bacteria in the soil environment and the activity of urease and catalase.

## SUMMARY

Soil is a specific component of the natural environment. A characteristic feature of soil is its variability, which affects the occurrence and abundance of soil microbial populations, including free-living bacteria of the genus *Azotobacter*. The abundance of *Azotobacter* spp. in soils varies widely, ranging from a few cells (cfu) to several hundred thousand cfu in 1 gram of soil. Differences in the abundance of *Azotobacter* populations are due to differences in the properties of the soils studied, as well as the type of agrotechnical treatments, fertilizers and plant protection products used. The occurrence of *Azotobacter* spp. is influenced by soil pH and the content of total nitrogen and organic carbon, indicating that soil fertility is as important as soil pH. There is a need to continue research on assessing the influence of soil properties on the occurrence of this group of bacteria both spatially and concerning selected ecosystems to obtain a complete picture of the ecological diversity within the population of bacteria belonging to the genus *Azotobacter*. In many countries, including Poland, monitoring of changes in the quality of the soil environment under the influence of agricultural and non-agricultural human activities is carried out, with the monitoring mainly concerned with the chemical properties of soils. Microbiological parameters of soils for monitoring should be characterized by their relevance to the proper functioning of soils and sensitivity to fluctuations in various environmental factors and by a relatively straightforward methodology for their determination. The specification of the environmental preferences of *Azotobacter* bacteria presented above indicates that their abundance can be a suitable parameter for monitoring the biological properties of soils, as they are sensitive to changes in the soil environment, and their detection and initial identification method on a selective nitrogen-free medium are simple and specific.

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received – 10 February 2023
revised – 13 March 2023
accepted – 5 April 2023

Author declares no conflict of interest.