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# Microorganisms in biostimulants

**Opinion of the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety**

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2017:16
Risk assessment of microorganisms in biostimulants.

Opinion of the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety
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Norwegian Scientific Committee for Food Safety (VKM)
Po 4404 Nydalen
N – 0403 Oslo
Norway

Phone: +47 21 62 28 00
Email: vkm@vkm.no

www.vkm.no
www.english.vkm.no

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## Microorganisms in biostimulants

## Authors preparing the draft opinion

Erik Joner, Eystein Skjerve, Leif Sundheim, Arne Tronsmo, Yngvild Wasteson

## Assessed and approved

The opinion has been assessed and approved by Panel on Biological Hazards. Members of the panel are: Yngvild Wasteson (chair), Karl Eckner, Georg Kapperud, Jørgen Lassen, Judith Narvhus, Truls Nesbakken, Lucy Robertson, Jan Thomas Rosnes, Olaug Taran Skjerdal, Eystein Skjerve and Line Vold.

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## Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

# Table of Contents

# Summary

In March 2016, the EU Commission presented a proposal for new regulations on fertilising material. The regulation includes product rules for a wide range of organic and inorganic products. Microbial biostimulants is one of the categories of products that are included. Biostimulants, in the draft EU regulation, are defined as fertilising materials that affect nutrient processes independently of the product's own nutrient content and with the purpose of improving nutrient utilisation, tolerance for abiotic stress or quality of the crop. Positive list in which species of these bacterial genera are listed: *Azotobacter* spp, *Rhizobium* spp., *Azospirillum* spp and Mycorrhizal fungi are a part of the regulation.

Since the import and use of these organisms are the responsibility of both the Norwegian Food Safety Authority and the Norwegian Environment Agency, they asked VKM to submit a joint report on effects on health (humans, plants and animals), biodiversity and dispersal, quality of agricultural land and on soil environment.

## Conclusions:

### Health risks

Based upon our literature review, we have found no indication of any specific diseases in plants, animals or humans induced by the discussed microorganisms. A few reported cases of human disease are caused through wound infections or injections in immunocompromised patients. These represent a situation where any microorganism may induce infections and is not specific for the agents discussed in this report. In summary, the risk of any disease caused by the discussed microorganisms is considered negligible.

### Environmental risks

In soil the biodiversity, competition, adaptation and functional redundancy of microorganisms are extremely high. This means that introduced microorganisms have a very small chance for establishing, and even less so for affecting biodiversity and soil functioning. Introduction of nitrogen fixing species or fungi that can transport P to plants (mycorrhiza) will lead to an increase in the primary production. However, even a large increased activity for these processes will not outcompete naturally occurring symbiotic N-fixation or growth of inherently non-mycorrhizal plant species. Thus, the risks associated with introduced non-pathogenic microorganisms are very low.

**Key words**: VKM, risk assessment, Norwegian Scientific Committee for Food Safety, Norwegian Environment Agency, Norwegian Food Safety Authority, regulation, fertilising material, *Azotobacter* spp, *Rhizobium* spp., *Azospirillum* spp, Mycorrhizal fungi, negative health effects, humans, animals, plants, environment

# Sammendrag på norsk

I mars 2016 kom EU-kommisjonen med et forslag til et nytt regelverk for gjødselprodukter. Regelverket omfatter produktregler for et vidt spekter av organiske og uorganiske produkter. Blant annet er det inkludert en varetype som kalles mikrobielle biostimulanter. Biostimulanter er i EU-regelverket definert som gjødselprodukter som påvirker næringsstoffprosesser uavhengig av produktets eget næringsinnhold men som har som formål å forbedre næringsstoffutnyttelse, toleranse for abiotisk stress eller kvaliteten på avlingen. Videre er det oppført en positivliste med arter fra følgende bakterie slekter, *Azotobacter spp, Rhizobium spp., Azospirillum spp* samt mycorrhizasopp*.*

Siden innførsel og bruk av disse organismene kommer under ansvaret for både Mattilsynet og Miljødirektoratet, er VKM bedt om å levere en felles rapport om effekter på helse (mennesker, planter og dyr), effekter på biologisk mangfold og spredning, og effekter på kvalitet på jordbruksjord og jordmiljø.

## **Konklusjoner**:

### Helserisiko

Basert på en gjennomgang av relevant litteratur har VKM ikke funnet noen indikasjoner på noen spesifikke sykdommer hos planter, dyr eller mennesker som er forårsaket av de mikroorganismer som er inkludert. Det er noen få rapporterte tilfeller av sykdom hos mennesker ved sårinfeksjoner eller injeksjoner hos immunkompromitterte pasienter. Dette representerer en situasjon hvor alle mikroorganismer kan forårsake infeksjoner og er altså ikke noe spesifikt for mikroorganismer som er omtalt i denne rapporten. Risikoen for enhver sykdom forårsaket av de diskuterte mikroorganismer anses som ubetydelig.

### Miljørisiko

Det biologiske mangfold av mikroorganismer i jord er ekstremt høyt. Det er også stor grad av konkurranse, tilpasning og funksjonell overlapping i jord. Dette betyr at introduserte mikroorganismer har svært liten sjanse til å etablere seg, og enda mindre til å kunne påvirke det biologisk mangfoldet og de fysiske forholdene i jord. Innføring av frittlevende N-fikserende bakterier eller sopp som kan transportere fosfor til planter (mykorrhiza) vil føre til økning i primærproduksjonen. Selv en sterk økning i aktivitet i disse prosessene ville imidlertid ikke konkurrere med naturlig forekommende symbiotisk N-fiksering eller vekst av stedegne plantearter som ikke danner mykorrhiza. Dermed er miljørisikoen forbundet med innførte ikke-patogene mikroorganismer svært lav.

# Background as provided by the Norwegian Food Safety Authority and Norwegian Environment Agency

In March 2016, the EU Commission presented a proposal for new regulations on fertilising material. The regulation includes product rules for a wide range of organic and inorganic products. Among other categories, it is a category called microbial biostimulants. Biostimulants, in the draft EU regulation, are defined as fertilising material that affect nutrient processes regardless of the product's own nutrient content and with the purpose of improving nutrient utilization, tolerance for abiotic stress or quality of the crop. The reason for excluding biotic stress in the definition is that there is a need for a clear distinction to the pesticide regulation.

In the draft regulation it is stated that an EU fertilising material may contain microorganisms, including dead or empty cells of microorganisms and non-harmful residual elements of the media that they were produced in, and which have not undergone any treatment other than drying or freeze-drying. Furthermore, there is a positive list where species of these genera are listed: *Azotobacter* spp, *Rhizobium* spp., *Azospirillum* spp and Mycorrhizal fungi.

For microbial biostimulants the following additional health requirements to prevent unwanted organisms are included:

a) *Salmonella spp*. shall be absent in a 25 g or 25 ml sample of the CE marked fertilising product.

b) *Escherichia coli* shall be absent in a 1 g or 1 ml sample of the CE marked fertilising product.

c) *Enterococcaceae* must not be present in the CE marked fertilising product by more than 10 CFU/g fresh mass.

d) *Listeria monocytogenes* shall be absent in a 25 g or 25 ml sample of the CE marked fertilising product.

e) *Vibrio* spp. shall be absent in a 25 g or 25 ml sample of the CE marked fertilising product.

f) *Shigella* spp. shall be absent in a 25 g or 25 ml sample of the CE marked fertilising product.

g) *Staphylococcus aureus* shall be absent in a 1 g or 1 ml sample of the CE marked fertilising product.

h) Aerobic plate count shall not exceed $10^5$ CFU/g or ml sample of the CE marked fertilising product, unless the microbial biostimulant is an aerobic bacterium.

i) Yeast and mould count shall not exceed $10^3$ CFU/g or ml sample of the CE marked fertilising product, unless the microbial biostimulant is a fungus.

# Terms of reference as provided by the Norwegian Food Safety Authority/ Norwegian Environment Agency

Since the import and use of these organisms are under both directorates responsibilities we kindly ask VKM submit a joint report with answers to all the questions. We ask VKM to consider how far down on the systematic level, in the classification of microorganisms, it is needed to assess in order to answer the different questions in the assignment.

The Food Safety Authority and the Environment Agency wants VKM to answer the following questions for the organisms listed in the positive list:

1. **Health Effects**
   1.1. Can the use of these organisms, as fertilising materials, cause adverse effects on plant, animal or human health?
   1.2. With the criteria for biostimulants given in the draft regulation, is there any risk that it may follow other organisms with the products that could possibly lead to adverse effects on plant, animal or human health?
2. **Effects on biodiversity and dispersal**
   2.1. Is it likely that the relevant organisms may spread to other non-treated areas?
   2.2. Can import and use of the relevant organisms cause adverse impacts on biodiversity?
   2.3. Are any of the respective organisms not to be regarded as alien species according to the definition in the Norwegian Nature Diversity Act § 3?
3. **Quality of agricultural land**
   3.1. Could the use of these microbial biostimulants lead to that the treated area have reduced ability to act as production soil in agriculture in short or long-term perspective?
4. **Effects on soil environment**
   4.1. Can the use of the respective organisms alter nutrient cycles in the earth, so that it becomes greater risk for loss of nutrients through air and water?

# 1 Introduction

**Why is it interesting to apply *Azotobacter* spp, *Rhizobium* spp. and *Azospirillum* spp. in agriculture?**

There is no lack of nitrogen on Earth, as our atmosphere contains 78 percent nitrogen ($N_2$). The problem is that no eukaryotic organism, neither fungi, plants nor animals can use nitrogen gas ($N_2$) as a nitrogen source. All these organisms must therefore be supplied with other nitrogen sources, called bound nitrogen, as organic nitrogen (amino acids, proteins and nucleic acids) or inorganic nitrogen as ammonia ($NH_4^+$), urea ($CO(NH_2)_2$) or nitrate ($NO_3^-$). Plants absorb bound nitrogen from the soil to produce vital nitrogen containing organic materials. At harvest most of this will be withdrawn from the field. This can in agriculture be replaced by adding manure or industrially produced nitrogen fertilizers, or the nitrogen can be made available for the plants by so-called biological nitrogen fixation.

**Biological nitrogen fixation**

Only a few bacteria (prokaryotes) can exploit $N_2$ as N-source in a process known as biological nitrogen fixation. In this process, $N_2$ gas in the atmosphere is first reduced to ammonia ($NH_4^+$), which then is incorporated into an amino acid, and then the -$NH_2$ group can be transferred to other amino acids or to a biosynthetic pathway for nucleotide synthesis. Nitrogen fixation is an energy intensive process, and therefore needs energy in the form of ATP.

N2 + 8 H+ + 8 e- + 16 ATP = 2 NH3 + H2 + 16 ADP + 16 Pi

This “bound” nitrogen in the bacteria can then be directly transferred to plants or is available after the nitrogen-fixing bacteria dies.

The nitrogen-fixing bacteria are divided in two groups, the free-living bacteria and the symbiotic nitrogen fixing bacteria that live in symbiosis inside the roots of plants where the bacteria benefit from ample energy supply and protection, and are therefore able to fix large amounts of nitrogen.

Globally, the most important system for biological nitrogen fixation is the cyanobacteria *Anabena azolla* that lives in symbiosis with the fern Azola in rice paddy fields. Before the rice growers plants rice in wetlands, they first cultivate the water fern Azola. The ferns are naturally infected with *A. azolla*, and the fern grows up to cover the rice fields. Then they plant young rice plants between the Azola. When the rice plants grow up and shadow the ferns, they will outperform Azola which dies. The bound nitrogen in the fern will leak out and be absorbed by the rice plants. This symbiosis produces so much bound nitrogen that for centuries this has been a sustainable system where they have been able to grow rice without external application of a nitrogen fertilizer.

The free-living nitrogen-fixing bacteria have a challenge, and that is to get enough energy to be able to fix nitrogen. The cyanobacteria are beneficial in terms of access to energy, in that they are photosynthetic and therefore have good access to energy in the form of carbohydrates produced from their own photosynthesis. The non-photosynthetic nitrogen-fixing free-living bacteria have to live in the rhizosphere, the area near the roots of the plants. In the rhizosphere there is more available energy than in root-free soil, because the plant roots excrete carbohydrates produced in the plants photosynthesis.

**Why is it interesting to apply mycorrhiza to agricultural soil?**

Mycorrhizal fungi are ubiquitous symbiotic fungi that colonize the large majority of vascular plants and provide them with an extended absorption system for mineral nutrients. In agricultural systems, many plants form mycorrhiza contributing to nutrient transport in many economically important crops and fodder plants, particularly P supply in highly weathered, P-fixing soils that dominate Mediterranean, sub-tropical and tropical regions. Indigenous mycorrhizal fungi are found in all natural soils, and only strongly degraded and artificial soils are void of these fungi. Addition of mycorrhizal fungi to functioning agricultural soil is therefore generally considered as futile, particularly in view of the high cost of such inoculation. The only rationale for doing so is if the introduced fungi can enhance the amount of mycorrhiza or outperform the indigenous fungi.

# 2 Literature

## 2.1 Pubmed search

The literature search was undertaken using the Advanced Search Builder provided by PubMed (www.ncbi.nlm.nih.gov/pubmed). The following search terms were used (Title/ Abstract):

- *Azospirillum*
- Mycorrhiza
- *Azotobacter*
- *Rhizobium*
- Biodiversity
- Negative plant health effect
- Human health
- Soil
- Fertiliser

Search strings were constructed by combining the search terms using the bolean variable AND and OR and is shown below. There was no restriction on language but search was limited to reviews and publications in the last 10 years.

((((((((azospirillum[Title/Abstract] OR mykorrhiza[Title/Abstract] OR azotobacter[Title/Abstract] OR rhizobium[Title/Abstract]) AND biodiversity[Title/Abstract]) OR negative plant health effect[Title/Abstract]) OR human health[Title/Abstract]) AND soil[Title/Abstract]) OR fertiliser[Title/Abstract])) AND Review[ptyp] AND "last 10 years"[PDat]) Filters: Review, 10 years

The search returned 209 articles.

## 2.2 Web of Science search

The literature search was undertaken using the Advanced Search Builder provided by Web of Science (https://apps.webofknowledge.com/WOS_GeneralSearch_input.do?product=WOS&search_mode=GeneralSearch&SID=P2QT8CDT3CW2URbYzU7&preferencesSaved=www.). The same search terms were used as in Pubmed search and the same limitations were applied.

The search returned 809 articles.

## 2.3 Relevance screening

The titles of all hits were scanned, and for those that were of potential relevance, the abstracts were also inspected. The relevance screening was performed by the members of the project group, independently. Citations were excluded if they did not relate to the terms of reference. The reference lists in selected citations were scrutinized to identify additional articles or reports, overlooked by the searches.

# 3 Hazard identification and characterisation

## 3.1 *Azotobacter* spp

*Azotobacter* is a well-known free-living genus of nitrogen-fixing bacteria. The first representative of the genus, *Azotobacter chroococcum*, was discovered and described in 1901 by Martinus Beijerinck. *Azotobacter*, is a genus of gram-negative bacteria belonging to the *Gamaproteobacteria*, which is a class of large free-living obligate aerobe nitrogen-fixing bacteria that is common in soil. Twelve species are described. *Azotobacter* spp. can form a resting structure called cysts that are resistant to desiccation, mechanical disintegration and UV and ionizing radiation. It is therefore possible to produce a very storage-stable product of *Azotobacter*. The contribution of fixed nitrogen from biological nitrogen fixation by *Azotobacter* in agriculture is probably very limited especially under our climatic conditions.

The literature search retrieved no documentation of specific negative health effects caused by *Azotobacter* spp. on humans, animals or plants. However, as a soil bacteria, *Azotobacter* spp. may accidentally contaminate for example open wounds and cause infections. This is not to be regarded as a specific characteristic of *Azotobacter* spp., but as a general feature of environmental bacteria and their ability to act as opportunistic pathogens.

One hazard of environmental bacteria is their ability to serve as a reservoir for antimicrobial resistance determinant. The occurrence of such determinants may jeopardize treatment of opportunistic infections caused by these bacteria, or the determinants may be transmitted to pathogenic bacteria. *A. chroococcum* has been shown to express resistance towards heavy metals and commonly used antibiotics after long-term application of industrial wastewater used as irrigation of wheat fields from where *A. chroococcum* was isolated from rhizospheric soil (Aleem, Isar, & Malik, 2003).

## 3.2 Mycorrhiza

In nature, most plants live in mutualistic symbioses with fungi. The symbioses are called mycorrhiza, and the fungi involved are called mycorrhizal fungi. The symbiotic advantage for these fungi is that they receive an abundant supply of carbohydrates from the photosynthesis of the host plant. In return, the fungi provide a greatly expanded absorption organ (fungal hyphae acting as a prolongation of the root system), that render uptake of water and inorganic nutrients, especially phosphorus, far more efficient. The mycorrhiza formation is initiated by chemotactic growth of a mycorrhizal fungus towards the plant roots. When it reaches the root, it grows into root cells (Endomycorrhiza) or makes a so-called mantle around the roots and a so-called Hartig net between epidermal and cortex cells (Ectomycorrhiza).

**Endomycorrhiza** comprise several different types formed by different fungi and host plants, the most common being so-called arbuscular mycorrhizas (AM) that can be found in over 80 % of surveyed herbaceous plants and many deciduous trees. The AM fungi belong to the *Glomeromycota*, one of seven currently recognized divisions within the kingdom Fungi. Approximately 230 species have been described, and several of them have been found to be universally distributed.

After contact between the fungus and a plant root, the fungus gently penetrates the root cell wall, but not the cytoplasmic membrane. Inside the cell, the fungus produces highly branched, bush-like organs called arbuscules that invaginate the plasmalemma (Figure 3-1). This creates a large surface and close contract between the plant and the fungus for effective symbiotic exchange.

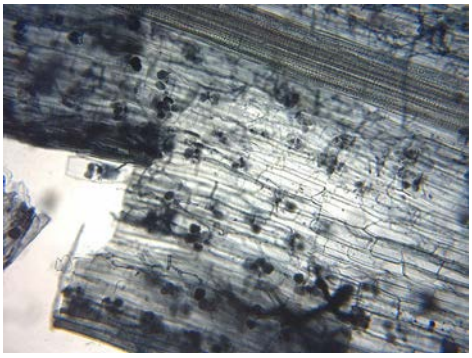

**Figure 3-1**. Light microscopy image of arbuscular mycorrhizal fungi inside a flax root. Note the branched structures (arbuscules) inside the root cells.

Arbuscular mycorrhizal fungi are obligate symbionts, meaning they cannot be cultured in the absence of plant roots. This implies that production of mycorrhizal inoculum is complicated and costly. Commercial inoculum has often been marketed as crude inoculum, which implies that the products consist of soil-like media used for growing the host plant, containing roots, spores and hyphal mycelium. Such production involves a certain risk for transferring plant pathogens together with the produced fungal inoculum. Yet, commercial inoculation products of pathogen-free arbuscular mycorrhizal fungi have been/are produced and/or marketed, in Norway, Europe and elsewhere.

Another common type of endomycorrhiza is the ericoid mycorrhiza which forms on plants of the genus *Ericaceae* (heather species including the plant genera *Calluna*, *Vaccinium,* and cultivated relatives like cultivated blueberries, rhododendrons and asaleas). These form symbioses with very different groups of scarcely investigated fungi belonging mainly to the *Ascomycota* (in the order Helotiales) and some to *Basidiomycota*. Some ericoid mycorrhizal fungal species from the former group have been cultured, while culturing fungi from the latter group has been difficult.

Commercial products of ericoid mycorrhizal fungi, based on axcenically grown fungi, have been/are produced and/or marketed, both in Norway, Europe and elsewhere.

**Ectomycorrhiza** are found on the roots of around 2 % of plant species, most of which are boreal and sub-arctic forest trees, including coniferous trees, and most members of a range of deciduous tree families like *Betula, Quercus, Fagus, Tilia, Acer, Alnus, Fraxinus*, and others. The mycorrhiza fungi are predominantly from the phyla *Basidiomycota* and *Ascomycota*.

The ectomycorrhiza fungi grow between the root cells, but they do not penetrate their cell walls. Instead, they enter between epidermal and cortical cells, resulting in a thickened root with restricted longitudinal growth. At the surface of these plant roots, the fungus forms sheath of mycelium, the mantle (Figure 3-2). From the mantle, the ectomycorrhizal fungi may spread its hypha into the soil and create an extended network of hyphae that absorb and transport immobile mineral nutrients (mainly N and P) and water.

Figure 3-2. Ectomycorrhiza formed by the fungus *Amanita* forming a typical mantel covering the root tips of its host plant.

Ectomycorrhizal fungi are, in some cases, very species-specific, and more than 6000 fungal species form ectomycorrhizas. Several of these are common mushrooms of both edible (*Boletus*, *Cantharellus*) and toxic genera (*Amanita*, *Cortinarius*). For other ectomycorrhizal fungi the host specificity can be low, as is commonly seen in early succession plant stands, where seedlings and young trees are quite unspecific in their association with fungal partners, and vice versa. Specificity is thus related to plant age, and often trees in a climax vegetation have fare more specific symbioses.

Commercial products of ectomycorrhizal fungi, based on axcenically grown fungi and targeting plant nurseries, have been/are produced and/or marketed, both in Norway, Europe and elsewhere. These mainly comprise early life stage colonizers, which are easy to grow axcenically in liquid culture media, and where produced inoculum has a relatively long shelf life. Later stage colonizing fungi are partly far more difficult to multiply.

The literature search retrieved no documentation of negative health effects from mycorrhizal fungi on humans, animals and plants.

## 3.3 *Rhizobium* spp.

Symbiotic nitrogen fixing bacteria supply bound nitrogen directly to the plants they live in symbiosis with. A very important system is *Rhizobium* and its relatives (*Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium* and *Photorhizoium*) that live in symbiosis with legumes. They are gram-negative *Alphaproteobacteria*. More than 75 species of *Rhizobium* spp., 31 *Bradyrhizobium spp.,* 21 *Sinorhizobium (Ensifer)* spp*., 32 Mesorhizobium* spp., 3 *Azorhizobium spp.* and one *Photorhizoium* species are described in List of Prokaryotic names with Standing in Nomenclature (LPSN).

These bacteria can live freely in the soil, but their nitrogen fixation activity is then low, partly because of limited access to energy. However, when they live inside the roots of leguminous plants, the nitrogen-fixing activity is considerable. In Norway, these bacteria live in symbiosis with clover, peas, beans and alfalfa and a variety of wild legumes.

There is high specificity between the *Rhizobium* species and leguminous plants. This means that when one introduces a new leguminous plant species, the right specific *Rhizobium* or relative species that can infect and make an effective symbiosis with the plant, may not be present in the soil. In this case, inoculation with the right species has to be done. If the plant species previously has been grown in that field, inoculation is not necessary, as there will be free-living *Rhizobium* or relatives present in the soil.

The symbiotic *Rhizobium* spp. and leguminous plants are very important for food and feed production in Norway, especially in organic farming. This system is so effective that not only is the clovers' own nitrogen needs fulfilled, but in a mixed population with clover and grass, surplus of bound nitrogen leaks out of the clover to the other plants in a mixed plant community. To get the full benefit of biological nitrogen fixation however, one must not fertilize with ammonium containing fertilizer because $NH_4^+$ inhibits nitrogen fixation.

Another cultivation system based on *Rhizobium* and legumes is called green manure. First a pre-culture with leguminous plants is grown. This is then ploughed down and the nitrogen will then be available for the next crop, for example wheat. This agricultural system is used in biodynamic and organic farming.

The *Rhizobiaceae* family in addition to the nitrogen-fixing legume symbionts also includes the genus *Agrobacterium*. *Agrobacterium tumefaciens* (syn. *Agrobacterium radiobacter, Rhizobium radiobacter*) can cause crown gall (tumours) on more than 140 plant species. Based on comparative 16S rDNA analyses, Young et al. (2001), suggested that *Agrobacterium* is an artificial genus and should be included in the genus *Rhizobium*.

*Agrobacterium tumefaciens* is regarded as an uncommon opportunistic pathogen in humans, particularly in immunocompromised patients. An infection caused by *A. tumefaciens* is associated with implanted intravascular devices, and case reports include endocarditis, bacteraemia and bloodstream infections. As *A. tumefaciens* is known as a plant pathogen in the agricultural industry, it is unlikely it will be intentionally included in any fertilising products.

The literature search retrieved no documentation of specific negative health effects caused by *Rhizobium* spp. for humans, animals and plants. The same general aspects as described for *Azotobacter* spp. applies to *Rhizobium* spp.

## 3.4 *Azospirillum* spp.

Another genus of bacteria, *Azospirillum,* a gram-negative rod to spirillum-shaped bacteria belonging to the *Alphaproteobacteria*, has gained much attention recently. Nineteen different species are described (Parte, 2014). *Azospirillum* spp*.* can form non-symbiotic association with various plants, in particular cereals and grasses. In warmer regions, such as in the Mediterranean climate, *Azospirillium* spp. have been shown to provide an important amount of nitrogen to plants. However, earlier experiments in Norway (Anon., 2017) have shown that in our cold climate, there seems to be a very limited contribution of fixed nitrogen to the plants from *Azospirillum spp*.

Soil bacteria like *Azospirillum* spp. may be found in environmental biofilms. Study by K. V. Kumar et al. (2015), demonstrated that *A. brasilense* co-aggregated with pathogenic leptospiral strains (the cause of leptospirosis), and that biofilms of leptospires in combination with *A. brasilense* showed high resistance to commonly used antibiotics. The study hypothesized that biofilm formation with *A. brasilense* protects the pathogenic *Leptospira* from adverse environmental conditions/stress.

The literature search retrieved no documentation of specific negative health effects caused by *Azospirillum* spp. for humans, animals and plants. The same general aspects as described for *Azotobacter* spp. applies to *Azospirillium* spp.

# 4 Exposure

## 4.1 Human and animal exposure

When they are in contact with soil, humans and animals are exposed to large numbers of indigenous *Azotobacter* spp., mycorrhizal fungi, *Rhizobium* spp. and *Azospirillum* spp. Uptake of these organisms could take place by ingesting soil or, for the bacteria, by contact with open skin wounds. Fungal propagules of mycorrhizas are too large to be relevant for uptake through skin (wounds). For farmers dealing with products containing high numbers of the organisms described above, exposure concentrations could be elevated if ingested or used without personal protection equipment. However, as none of these organisms are regarded as pathogens, they do not contain known pathogenicity factors such as specific adherence factors or toxins.

Many soil bacteria and fungi are regarded as opportunistic pathogens for humans and animals causing infections if introduced to open skin wounds or into an animal or human host by different devices like artificial valves, catheters etc. As these soil bacteria also often thrive in aquatic environments, hospital patients may become exposed through contaminated devices if hospital hygiene is inadequate.

The rhizosphere comprises large populations of different microorganisms (Naamala, Jaiswal, & Dakora, 2016). If new populations are to be added, these should be able to compete with the resident flora in order to establish themselves. Resistance to antibiotics that may be naturally produced by the residential flora may therefore be regarded as a desirable trait for the ”newcomers”. However, it is of the outmost importance that the resistance characteristics are intrinsic, as extrinsic resistance characteristics may be transmitted to other bacteria and thus contributing to the global emergence of antimicrobial resistance.

## 4.2 Plant exposure

Plants are commonly exposed to *Azotobacter* spp., mycorrhizal fungi, *Rhizobium* spp. and *Azospirillum* spp.

Bacteria in the genus *Azotobacter* are aerobic and free-living in the soil. By binding atmospheric nitrogen and releasing ammonium ions in the soil, they have a positive effect on plant growth. *Azotobacter* spp are widespread in soil and globally distributed.

There are numerous, scientific reports on the beneficial effects of mycorrhizal fungi on plant health. Arbuscular mycorrhiza is the most common form for mycorrhiza, and this symbiosis is found on the roots of 80% of all vascular plants. The mycorrhizal fungi penetrate the roots of vascular plants to form arbuscules inside the cortical cells. These specialized fungal structures aid plants in the uptake of phosphorus, sulphur, nitrogen and micronutrients from the soil. Ectomycorrhiza is formed by fungi that do not penetrate the cell wall of their host plants. The fungal hyphae are branched into a latticework between epidermal and cortical root cells. A dense hyphal mantle, up to 40 µm thick, surrounds the root surface. The fungal mantle aids the host plant in uptake of minerals and water, while the fungus is supplied with carbohydrates from the plant. Ectomycorrhiza is common on forest trees in temperate climate ecosystems (Cameron, Neal, van Wees, & Ton, 2013; Garmendia, Goicoechea, & Aguirreolea, 2005).

Bacteria in the genus *Rhizobium* form endosymbiotic, nitrogen-binding associations with legumes. These bacteria have a positive effect on the hosts plant by providing organic, nitrogenous compounds to the plant. *Rhizobium* spp. are globally distributed, including both arid and arctic climates (Dutta, Mishra, & Kumar, 2008; Gourion, Berrabah, Ratet, & Stacey, 2015; B. S. D. Kumar, Berggren, & Martensson, 2001).

Several *Azospirillum* spp. are commonly associated with plant roots. Most studies of these bacteria have been conducted on cultivated, gramineous plants. There are reports that

*Azospirillum* spp. may reduce the susceptibility of plants to plant pathogens (Bashan & de-Bashan, 2010).

## 4.3 Summary of exposure

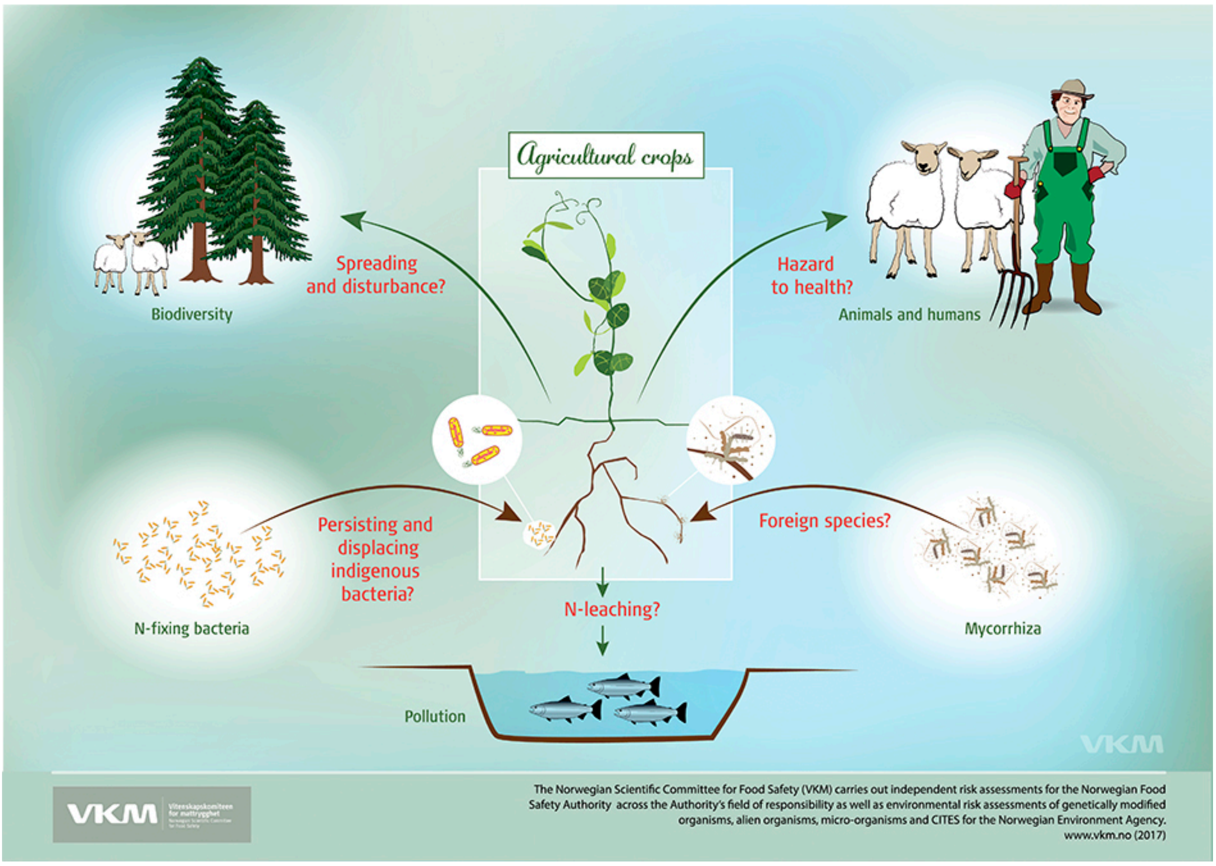


Figure 4-1 Exposure pathways for microorganisms in biostimulants. (Eric Joner and Monica A. Widing/Brace)

# 5 Risk characterisation

**Health risks**

Based upon our literature review, we have found no indication of any specific diseases in plants, animals or humans induced by the discussed microorganisms. In summary, the risk of any disease caused by the discussed microorganisms is considered negligible.

**Environmental risks** include deterioration of natural and man-made (e.g. agricultural) habitats with respect to productivity, biodiversity and quality of ecosystem services. Directly involved ecosystem services from soil microorganisms are N-fixation and mycorrhizal P transport (and secondary effects of mycorrhizal fungi on plants and soils). Other relevant ecosystem services from soil microorganisms are organic matter mineralization, mineralization of plant nutrients, ammonification, nitrification, denitrification, methane consumption, degradation of harmful natural and man-made chemicals, etc.

As is the case for risk characterisation related to human health, environmental risk is composed of a hazard component multiplied by the probability of occurrence or exposure. Foreign organisms represent a hazard only if they are likely to suppress indigenous organisms in terms of reducing their abundance, functional roles or fitness. This can occur for introduced plant species (black-listed species) or organisms that multiply and spread easily due to no or few competitors and/or predators. In soil the biodiversity, competition, adaptation and functional redundancy of microorganisms are extremely high. This means that introduced microorganisms have a very small chance for establishing, and even less so for affecting biodiversity and soil functioning. This is mainly due to the fact that introduced species are less well adapted to local conditions of nutrient availability, pH, temperature, competitor/predators, etc., and the fact that all exploitable niches are already occupied by well adapted indigenous microorganisms. Thus the probability part of risks associated with introduced non-pathogenic microorganisms is very low. The hazard represented by introduced species establishing and fixing N (for introduced N-fixing bacteria) or transporting P to plants (mycorrhiza), has few imaginable negative effects, as a small to moderate increase in these processes would merely lead to a small increase in primary production (Klironomos, 2003; Moora et al., 2014; Schwartz et al., 2006; Sieverding & Oehl, 2005; van der Heijden, Boller, Wiemken, & Sanders, 1998). Even a large increased activity for these processes would not be able to outcompete e.g. symbiotic N-fixation or growth of inherently non-mycorrhizal plant species. Thus, the environmental risks associated with introduced non-pathogenic microorganisms is very low.

# 6 Uncertainties and data gaps

The degree of confidence in the final estimation of risk depends on the variability, uncertainty, and assumptions identified in all the previous steps. Discrimination between uncertainty and variability is important in subsequent selection of risk management options. Biological variation includes the differences in virulence that exist in microbiological populations and variability in susceptibility within the human, animal and plant population and particular sub-populations. (http://www.fao.org/docrep/005/y1579e/y1579e05.htm).

In this assessment, a number of uncertainties related to the risk of negative health effects have been identified. Many of these uncertainties overlap with the data gaps and are therefore described together.

The uncertainties identified are as follows:

- Negative effects can never be excluded, and there is always an uncertainty linked to a lack of information. However, in a risk assessment we have to deal with available information. In the light of the massive exposure of plants, animals and humans to indigenous equivalents it would be highly surprising if scientists have not detected these theoretical side-effects already.
- We consider that the lack of information about any negative health effects reflects a situation where these most likely do not exist or at worst are only of marginal importance.
- Interactions between microorganisms in the environment is an evolving situation. The research area is in its infancy, new tools are constantly developing and new knowledge that can influence the assessment and understanding can become available.
- Using existing methods, the literature support the conclusion that these biostimulants are safe to use. However, using new methods for characterization of local microbiomes (genomics) may demonstrate local changes to the composition of bacteria in the affected area.

# 7 Conclusions (with answers to the terms of reference)

1. **Health Effects**

1.1. *Can the use of these organisms, as fertilising materials, cause adverse effects on plant, animal or human health?*

All microorganisms on the positive list are regarded as beneficial microorganisms for plant growth by providing nutrients and water to the plants. There are also many examples of these beneficial microorganisms increasing the disease resistance in plants. We have not been able to find any publications that document negative effect by these microorganisms on plants, animals or human health.

1.2. *With the criteria for biostimulants given in the draft regulation, is there any risk that it may follow other organisms with the products that could possibly lead to adverse effects on plant, animal or human health?*

The hygienic demands for the products described in the regulation are very strict. To fulfil these hygienic requirements the producer of these products has to propagate them under uncontaminated conditions. This is also the case for the obligate biotroph, mycorrhizal fungi. Therefore, it is unlikely that the products will contain harmful organisms that can cause negative effects on plants, animals or human health, if the hygienic demands are fulfilled.

2. **Effects on biodiversity and dispersal**

2.1. *Is it likely that the relevant organisms may spread to other non-treated areas?*

All these beneficial microorganisms are natural inhabitants of soil in Norway. They have been spread and will continuously be spread during different agricultural operations. Also, inoculated microorganisms will be spread, if they are able to grow and multiply in the soil. That will however depend on how competitive they are. Many inoculation experiments in agricultural soil have proved that it is difficult for the introduced microorganisms to compete with the natural microflora and then multiply in the soil. To do so, they must have ecological fitness for Norwegian soil and climate. Introduced microorganism, selected for other climatic zones or soil types, may therefore not be able to persist and spread.

Introduced *Rhizobium* ssp. and their relatives may however have an advantage after introduction of a new legume species, if there is no *Rhizobium* spp. present that is able to infect and make nodules on a newly introduced legume. This has been a successful agronomic practice in Norway for several decades.

2.2. *Can import and use of the relevant organisms cause adverse impacts on biodiversity?*

As all the microorganisms on the positive list occur naturally in Norwegian soil, it is very unlikely that they will have unwanted effects on the biodiversity. The only negative effect that could be foreseen is if this beneficial microorganism is transferred to an area where one wants to have very low fertility.

2.3. *Are any of the respective organisms not to be regarded as alien species according to the definition in the Norwegian Nature Diversity Act § 3?*

All the microorganisms on the positive list are regarded as natural inhabitants in Norwegian soil.

## 3. Quality of agricultural land

3.1. *Could the use of these microbial biostimulants lead to that the treated area have reduced ability to act as production soil in agriculture in short or long-term perspective?*

No negative effect is anticipated on a short-term and long-term perspective.

## 4. Effects on soil environment

*4.1. Can the use of the respective organisms alter nutrient cycles in the earth, so that it becomes greater risk for loss of nutrients through air and water?*

Use of any of these beneficial microorganisms can potentially improve the nutrition status of the soil and be beneficial for plant growth and plant health. There will be no increased probability for loss of plant nutrients compared to untreated soil, except the intended effect of enhanced N-fixation that may increase plant N nutrition and later moderately enhance soil N levels. This N enrichment is equivalent to fertilization with mineral N and a necessary means to produce crop plants. Both means of N enrichment for enhanced plant productivity result in that residual N in soil may leach out of the soil or be lost by denitrification. Leaching can in this case potentially pollute rivers and streams. Mycorrhizas do not produce any nutrients, they merely scavenge soils for soluble nutrients and thus reduce the risk of pollution from run-off.

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