Literature Review

Summary of Microbiology

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Executive Summary

The applicant seeks to address the criteria outlined in section 36 of the Hazardous Substances and New Organisms (HSNO) Act 1996 with regard to an application for full release of three microbial species new to New Zealand which is foliar applied to plants to facilitate in nitrogen fixation.

A review of scientific literature has investigated the effect of these microbes with respect to potential deterioration or displacement of native fauna and flora, pathogenicity and toxicity nature of these microbes. The majority of literature written, specific to these bacteria, indicate the significant impact they have within agricultural applications as mutual symbiotic organisms which contribute not only as significant nitrogen providers to the plant (via nitrogen fixation) but with other beneficial effects being recorded such as increased plant growth, root formation and plant health.

Additional science has shown too that these microbes when introduced to an exotic environment are subject to significant competition from native soil species which eventually destroy the introduced species. This is also evident in practice with inoculations requiring repeated applications to crops to ensure effectiveness.

1 Introduction

1.1 Aim

The applicant is applying for approval from the Environmental Protection Authority (EPA) New Zealand for the full release of three microbiological species which are new to New Zealand. This literature review is intended to be a supporting document to the application. The aim of this document is to address the minimum standards in section 36 of the Hazardous Substances and New Organisms Act 1996[1] (HSNO Act) with regards to the three nitrogen fixing microbes, highlighting previous scientific research and findings on these organisms.

1.2 Minimum Standards

Section 36 of the Hazardous HSNO Act states the criteria which must be met for an application to be approved for the release of a new organism into New Zealand. This states;

> *The Authority shall decline the application, if the new organism is likely to—*
>
> *(a) cause any significant displacement of any native species within its natural habitat; or*
>
> *(b) cause any significant deterioration of natural habitats; or*
>
> *(c) cause any significant adverse effects on human health and safety; or*
>
> *(d) cause any significant adverse effect to New Zealand's inherent genetic diversity; or*
>
> *(e) cause disease, be parasitic, or become a vector for human, animal, or plant disease, unless the purpose of that importation or release is to import or release an organism to cause disease, be a parasite, or a vector for disease.*

For the sake of this literature review it is to be understood that condition *(a)* is related to the displacement of the natural soil microbe populations, conditions *(b)* and *(d)* can be combined with regard to the deterioration of plants and natural plant health and development, condition *(c)* is in regard to the toxicity of the microbes to human health and condition *(e)* is related to the pathogenic nature of the introduced microbes to plant and animal (including human) health.

1.3 Microbiology

There are three microbial species from three different genera under scrutiny here. Since the majority of scientific publications dealing with the species of interest focuses on topics such as mode of action, beneficial effects, interaction with plants and taxonomy and less on the potential hazards or detrimental effects related to these microbes the scope of this study is expanded to include not only the three species of interest but the genera they represent.

The three genera therefore investigated are:

1. Azospirillum
2. Azorhizobium
3. Azoarcus

Of these three genera, Azospirillum has had the most cover in literature with regard to its use in Agriculture and other scientific studies, followed by Azorhizobium and then Azoarcus with significantly less exposure. The fact that Azoarcus is much less studies could possibly be attributed to the preferential host plant growing in the tropics, a factor that is likely to limit the survival rate of the organism in more temperate climates.

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[1] http://www.legislation.govt.nz/act/public/1996/0030/latest/DLM383527.html?search=qs_act_new+organism_resel&p=1#DLM383527

This literature review will address, for each genus the conditions listed in Section 36 of the HSNO Act as mentioned above.

1.4 Definitions

Diazotroph - are bacteria that fix atmospheric nitrogen gas into a more usable form such as ammonia.

Free living diazotrophs - are often found in the rhizosphere and in the intercellular spaces of several plants

Symbiotic diazotrophs – include the associations in which an organism lives on or in its host (or plant). Often in a close and long-term interaction between the different species.

Rhizobium - form an endosymbiotic nitrogen fixing association with roots of legumes. The bacteria colonize plant cells within root nodules; here the bacteria convert atmospheric nitrogen to ammonia and then provide organic nitrogenous compounds to the plant. The plant provides the bacteria with organic compounds from the photosynthesis process.

Endophyte – microbes which live within a plant for at least part of its life without causing disease. They may benefit host plants by preventing pathogenic organisms from colonizing them.

2 Literature Review

2.1 Introduction

2.1.1 The Nitrogen Cycle

The nitrogen cycle is at the core of our agricultural industry where plants rely on the chemical or biological conversion of nitrogen gas (N_2) present in the atmosphere into a more readily available form for uptake by plants namely the nitrate ion (NO_3^-) or ammonium ion (NH_4^+). Animals obtain the nitrogen required for cell growth by the consumption of plants and return excess nitrogen to the soil in the form of urea. Apart from water, nitrogen is one of the most important nutrients that can limit crop yields in soils (Saikia and Jain, 2007).

The most important process of accumulating nitrogen (N) in an organic form in the soil is through nitrogen fixation (Mengel and Kirby, 1987) carried out by soil micro-organisms present in the soil, around plant roots and even within the plants themselves. It has been reported that on a global scale up to 3 x 10^9 tonnes of N per year is fixed by soil microbes (Zahran, 1999). Globally, this accounts for up to 60% of the total nitrogen available to plants. On an average NZ dairy farm however, nitrogen fixation makes up only 40% of the nitrogen supplied to plant growth compared to chemical fertiliser N inputs which is around 50%.

2.1.2 Microbiology and N-fixation

Microbiological processes play a significant role in the various stages of the nitrogen cycle i.e. the microbial enzyme ‘urease’ facilitates the conversion of urea to ammonium, nitrifying bacteria (nitrosomonas and nitrobacter) convert the ammonia to nitrite and nitrate respectively while denitrifying bacteria are responsible for the eventual conversion of nitrate to nitrous oxide and nitrogen.

The bacteria responsible for fixing (assimilating) nitrogen from the atmosphere are the diazotroph bacteria. Some of these are free living nitrogen fixers in the soil but in most cases the nitrogen fixers live in the rhizosphere (soil fraction affected by root activities see Figure 1) interacting with the surface of plant roots (symbiotic and associative) or are present within the root cells themselves (endophytic). Of particular importance to the agricultural industry are the Rhizobium bacteria which live in symbiosis with leguminous plants such as clover where root nodules are formed that can be seen with the naked eye.

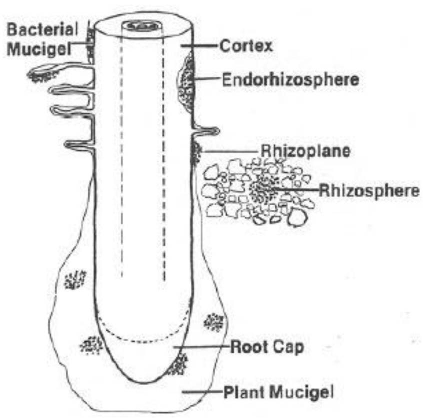

Figure 1: Diagram showing the area of soil around a plant root system as the rhizosphere.

The rate of natural nitrogen fixation is dependent on environmental conditions including pH, moisture, salinity and temperature (acidic conditions and low temperatures are not conducive to high rates of biological fixation [Mengel and Kirby, 1987]).

Effective atmospheric nitrogen fixation is often measured by the production of the enzyme nitrogenase which is used by the N-fixing bacteria to convert nitrogen (N_2) to ammonia (NH_3).

2.2 The Three Genera

2.2.1 Azospirillum

Azospirillum bacteria was re-discovered (and subsequently re-named from Spirillum to Azospirillum [Umali-Garcia, 1980]) in the 1970's and has the reputation of being one of the most studied plant – bacteria associations (Bashun and Holguin, 1997) with regard to agricultural benefits particularly in non-leguminous crops, e.g. wheat, maize etc (e.g. Ganapathy and Saval, 2006, Vazquez et al. 2000, Saikia and Jain, 2007). Studies have shown it to be closely associated with the root structure of plants but it differs from the symbiotic rhizobium species of bacteria (Umali-Garcia, 1980) and are therefore known as *associative diazotrophs* (Tilak, 2005). The nitrogen fixing Azospirillum bacteria mainly act by influencing the morphology, geometry and physiology of the root system (Barea, 2002) compared to other diazotrophs which are symbiotic micro organisms (mutual give-take relationship with a plant) or endophytic micro organisms which colonise within the plant tissue.

2.2.2 Azorhizobium

The Azorhizobium genus was classified over 20 years ago by Dreyfus et al. (1988) after the microbe was isolated from its host plant *Sesbania rostrata*. This plant is well known for its high N-fixing ability due to the formation of nodules on both the root and stems of the plant and therefore is used as a green manure particularly in rice paddys (Ladha et al. 1989). Azorhizobium have the unique ability to fix nitrogen both as a free living organism and in a symbiotic interaction with its host plant (Lee et al. 2008). It is this dual N-fixing ability which sets it apart from other Rhizobia organisms.

2.2.3 Azoarcus

Azoarcus is endopyhtic bacteria of which was isolated from the roots of Kallar Grass in Pakistan (Hurek et al. 1994). It is observed to have extremely active nitrogenase activity (Kennedy et al. 1997, Krause et al. 2006), which is the enzyme used to convert atmospheric nitrogen gas to ammonia.

2.3 Displacement of Native Species - Condition (a)

The science of artificial soil inoculation began over 100 years ago, although the effectiveness of successfully populating an ecosystem is thwarted by the competition with the native microflora (microbiology) already present in the soil (Biro, et al. 2000). Bashan (1999) published a review of the studies done on Azospirillum and its persistence in soils and stated that soils react like a biological buffer where a change in its microbiological diversity is only temporary since the native microbes prey on the introduced species. This paper showed that Azospirillum when inoculated onto various soils under various conditions can last from 9 days in the short term to 14 months (at this stage in very low numbers) in the long term whereafter a very sharp decline in population is generally noted.

Another study of Azospirillum which was heavily inoculated onto the soil at weekly applications for 4 weeks showed an initial decline in the native microbial population due to the abundance of Azospirillum present in the soil. The inhibitory effect however only lasted until 2 weeks after inoculation as the native populations re-established themselves to the same level as before inoculation (Bashan 1999). These effects are also observed with many specific microbial products where re-inoculation is required at intervals of commonly two months after initial inoculation due to native population competition.

Bashan et al (1995) found that in 23 different soil types the inoculated Azospirillum populations generally declined with time. Two main factors were found to be directly related to these microbes survival. These were (i) increasing levels of $CaCO_3$ in the soil and (ii) increasing sand fractions in the soil both having a detrimental effect on survival. In addition to this the study found that after removal of plants (i.e. due to harvest) any remaining residual material supported the microbes for only a few weeks before microbe die-off occurred since the microbes live in association with living plants.

As with Azospirillum, soils inoculated with the Azorhizobium microbe have shown a decline in the introduced species population over time (Ladha et al. 1989). This is in agreement with other studies of Azorhizobium inoculation into soils which show the requirement for numerous inoculations of the microbe over time for desired plant effects to be maintained (e.g. Kennedy et al. 1997) suggesting an obvious die-off / competition factor of the introduced species compared to the native species.

The endopyte Azoarcus has been found to live successfully within plants but do not survive in soil (Reinhold-Hurek and Hurek, 1998). Since this microbe originates from the tropics it is unlikely that it would survive the lower temperatures of New Zealand for a lengthy period of time.

2.4 Deterioration of Natural Habitat - Conditions (b) and (d)

The three microbes are used to enhance plant growth and development and therefore are not considered to be deleterious to the natural habitat. Endopytes such as Azospirillum and Azoarcus, and Rhizobia such as Azorhizobium are considered capable of increasing yields, removing contaminants, inhibit pathogens and contribute to nutrient (i.e. nitrogen) levels (Rosenblueth and Martinez-Romero, 2006, Hardoim et al. 2008, Saika and Jain, 2007).

A study of an Azospirillum inoculant on plants in mine tailings in Arizona showed a significant positive impact on plant growth with particular emphasis on enhancing (and not deteriorating) the natural rhizosphere microbial community, by improving plant root development and root exudations (de-Bashan, 2010). This indirect stimulation of the ecology of the rhizosphere has been shown in other studies as well (Bashan and Holguin, 1997).

It seems Azorhizobium microbes also have the effect of being non-damaging but instead facilitates significant increases in plant yield (Ponnuswamy et al. 2002) and nitrogen content. Again this appears to be predominantly due to the stimulation of root development and the formation of large numbers of short lateral roots (Sabry et al. 1997, Kennedy et al. 1997) which are used by the plant to extract water and nutrients from the surrounding soil.

Similarly, studies on the effect of Azoarcus introduced to plant species show only positive results with no deterioration but instead facilitating yield increases and higher nitrogen provision to the plant (i.e. Jilani et al. 2007, Krause et al. 2006). Hurek et al. (1994) also suggest Azoarcus may also contribute to enhancing a plants mineral uptake and improving plant-water relationships potentially via root stimulation.

A controlled study (Dowsett et al. 2010) was undertaken in 2010 by AgResearch with regard to addressing the minimum standards (a) and (e) in section 36 of the HSNO Act for the three microbes being discussed. This study concluded that the three microbes had no detrimental effects on the growth and development of the selected native plant species nor did the microbes affect the microbial diversity in the soil at all.

2.5 Adverse Human Health Effects - Condition (c)

Little is published regarding the human toxicity of the three micro organisms. However, experts in the field of microbiology have deemed these organisms safe to use. In searching the international culture collections of the three genera, few organizations published the risk levels of the micro organisms. Table 1 summarises the risk level of each microbe from a couple of sources.

Table 1: Culture Collection Stated Risk Levels.

Genus	American Type Culture Collection (ATCC)	Belgian Coordinated Collections of Micro-organisms (BCCM)
Azospirillum	Biosafety Level 1[1]	Biohazard Group 1[2]
Azorhizobium	Biosafety Level 1	Biohazard Group 1
Azoarcus	Biosafety Level 1	Biohazard Group 1

[1] Biosafety 1 = microorganisms not known to cause disease in healthy adult humans. (http://www.atcc.org)

[2] Biohazard 1 = organisms that are unlikely to cause human disease. (http://bccm.belspo.be)

This information indicates the low risk which the international community associates with the micro organisms concerned. In addition to these low risk classes above, it can be deduced from a review of the previous and current research in which these organisms were studied that there is little or no risk of adverse human health effects. All three micro-organisms have been used in human food (production systems) research applications (i.e. wheat, corn, rice, alfalfa, legumes etc) for many years and thereby it can be deduced these organisms are safe and provide minimal risk to human health. Many biological products such as these are internationally registered as organic products by organisations such as NASAA (Australia), OMRI (USA), and Soil Association (UK).

2.6 Cause or Carry Disease - Condition (e)

This review was unable to identify studies which have specifically focused on the pathogenic nature of Azorhizobium (apart from the biosafety levels outlined in section 2.5 above). However studies which have concentrated on this microbe describe it as one which is able to live in a mutual symbiotic relationship with its host (plant). There are no reports in the literature that we are aware of that report any signs of pathogenicity in plants or other organisms.

Azospirillum are known to benefit plant development and even yields under certain conditions (Barea, 2002, de-Bashan et al. 2010). The Azospirillum microbe interacts with native mycorrhizal fungi in the soil and enhances this symbiotic fungi's presence in the soil which in turn reduces damage by soil borne plant pathogens increasing plant health and resistance to disease (Vazquez et al. 2000, Pozo et al. 2002). In another study Bashan (1998) was also able to show the safety of Azospirillum inoculation where he compared the effects on plants using this microbe alongside known plant pathogens. In each case the Azospirillum inoculated plants failed to show any disease symptoms or pathogenic reaction. In comparison these plants all had increased root hairs and greater lateral root production.

Hurek et al. (1994) undertook a pathogenicity test to determine what reaction a plant would have if Azoarcus was applied. This was done alongside a known plant pathogen. Results showed the plant did not respond or show any signs of leaf damage for up to 14 days after application of these two organisms while without Azoarcus spp. symptoms developed after two days suggesting Azoarcus is not phytopathogenic. Instead it showed the ability to protect the plant against some pathogens.

3 Conclusion

In taking into consideration the large amount of international work undertaken in agricultural systems on the three microbes over the past 30 plus years, and reviewing the mechanisms and effects on plants by which these microbes interact with, it can be summarised that the potential for any detrimental effects on New Zealand native flora and fauna is extremely low. The detailed study by AgResearch (Appendix 1) confirmed that the microbe species have no deleterious effects on native plants or microbial populations. In reviewing the literature it can be surmised that in fact the opposite is true, with positive effects of plant health, plant growth and good soil microbial competition taking place within any environment these bacteria are introduced in to. Natural processes such as soil microbe competition, plant removal and environmental conditions (temperature, pH etc), coupled with experience from agriculture applications also ensure that displacement of native microbes does not take place.

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