March 6, 2015

MEMORANDUM

Via Electronic Mail

To: De Ceuster N.V.
Fortsesteenweg 30
2860 Sint-Katelijne-Waver
Belgium

10.2.e

From: [redacted]

Re: Request to provide regulatory support for the pesticide risk assessment of the active substance Pepino mosaic virus, strain CH2, isolate 1906, in the form of technical (microbiological) expertise focused on the review of pathogenicity of coliforms.

De Ceuster N.V., Fortsesteenweg 30, 2860 Sint-Katelijne-Waver, Belgium (also “you” and “the Client”) requested that ENVIRON International Corporation (hereafter "ENVIRON" also "we" and "us") provide scientific and technical assistance in conducting a literature review and assessment, to provide data to support observations and conclusions on the potential pathogenicity of pesticide samples tested for indicator coliform bacteria. The samples were prepared from [redacted] the active substance *Pepino mosaic* virus, strain CH2, isolate 1906.

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In this phase, ENVIRON was specifically requested to provide data on the implications of using observations from indicator microorganism assessments, including evaluations of bacterial coliforms (e.g. total coliform and fecal coliform (e.g. *Escherichia coli* (*E. coli*)), as an indication of the potential presence or absence of pathogens in analytical samples, in response to data gaps noted by the European Food Safety Authority (EFSA) during their regulatory approval of the pesticide active substance *Pepino mosaic* virus, strain CH2, isolate 1906.

Questions Presented

De Ceuster N.V. asked ENVIRON the following questions:

1. Is the presence of coliforms other than *Escherichia coli* (*E. coli*) an indication of pathogenicity?
2. Does the detection of fecal coliforms in a sample imply pathogenicity of the sample?
3. Can total coliforms and fecal coliforms be detected in samples of plant endophyte extracts?
4. Is *E. coli* the only human pathogenic fecal coliform?

Brief Answer

Based on our research and review, ENVIRON determined that plant endophyte extracts could contain a mixture of benign or non-pathogenic bacteria and coliform bacteria. Although the majority of bacteria that test positive to coliform assays of horticultural produce are benign or non-pathogenic, due to the inherent existence of plant endophytes some extracts of plant endophytes could contain microorganisms of human health concern including pathogenic coliforms. However, research has shown that most endophytic bacteria exhibit characteristics that make them beneficial to the host rather than harmful or pathogenic.

It was also determined that the detection of total coliforms is only suggestive of the potential presence of pathogens in a sample. A positive coliform result does not confirm the presence of pathogens in a sample. However, the detection of fecal coliforms in a sample indicates that pathogenic coliforms could most likely be detected in the sample. Substantiating or confirming the presence or absence of specific pathogenic organisms in a sample, requires testing for specific known pathogens that may or may not belong to the coliform group. The process of performing confirmatory tests for all known pathogens associated with a particular sample or formulation may be impractical and time-consuming. Hence, it is commonplace to assess key indicator microorganisms such as fecal coliform bacteria as an indication of possible pollution or microbial pathogenic contamination of a sample. Because fecal coliform bacteria such as *E. coli* are not naturally free living in the environment, but originate from the intestines (gut) of warm blooded animals and humans (hosts), these coliforms when present in the host can be transferred into an environment suitable for their growth such as soil, water and food via excretion by the host. Not all strains or types of fecal coliforms are pathogenic (cause disease), although some are; hence their detection in an environment or medium is not definitive or confirmatory of pathogenic contamination, but only an indication of the possibility for pathogenic contamination of that environment. Pathogenicity can only be confirmed if a known pathogenic coliform is isolated from the environment or test substance.

Although not all strains of *E. coli* are pathogenic, the rationale behind the general acceptance of a positive fecal coliform test confirming the presence of pathogenic strains of *E. coli*, e.g. *E. coli* O157: H7, as an indication of the likely pathogenicity of a test sample is because *E. coli*:

- Unlike other bacteria, coliforms are not found free living in the environment, but originate from feces or the gut of warm blooded animals, hence it is generally accepted as a good indicator of the presence of fecal coliforms in a sample;
- Can be rapidly assessed in a test sample;
- Is a known human pathogen that is the most abundant coliform bacteria found in human feces, hence its presence is an indication of the possibility that the sample has been contaminated with feces and as such other external pathogenic coliforms of fecal origin could also be present in the sample; and
- Most pathogens including pathogenic *E. coli* strains originate from fecal or waste matter which tends to harbor coliforms.

For this reason a sample that is negative for the presence of a pathogenic strain of *E. coli,* the most abundant fecal coliform bacteria, is most likely pathogen-free but not definitively pathogen-free.

The samples tested were batches of [redacted] the active substance *Pepino mosaic* virus, strain CH2, isolate 1906. *E. coli* was not detected in any of the five batches of samples analysed.

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Since *E. coli* is the most commonly tested human pathogen belonging to the fecal coliforms or thermotolerant coliforms group, a test for *E. coli* is generally accepted as an indication of a

sample free of fecal coliforms and associated pathogenic coliforms. It is well described in literature that the fecal coliforms assay is not a reliable indicator of the fecal contamination of plant foods including vegetables. This is because some bacteria detected as fecal coliform bacteria are not of fecal origin, but are rather plant associated bacteria living on the surface of plants (epiphytes) or bacteria living within the plant tissues (endophytes). It is therefore hypothesized that the coliform bacteria detected in the sample are plant associated bacteria (endo- and epiphytes).

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To further test the hypothesis that extracts from plant bacterial endophytes could test positive for coliforms but be devoid of pathogens, five unique batches [redacted] the active substance *Pepino mosaic* virus, strain CH2, isolate 1906 were analyzed to quantify and identify the coliforms in the samples. The coliforms found were identified as: [redacted]

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- [redacted] an endophytic symbiont that can be used as a plant growth promoter and has potential use as a biocontrol agent for several pathogens in agriculture.
- [redacted] a bacterium commonly found in the environment with potential use in phytoremediation of polluted environments.
- [redacted] an epiphytic bacterium found on the surface of the leaves and is well known for the biological control of several diseases in pome fruits.

These bacteria are described in literature as endo- and epiphytic bacteria, hence their presence in the samples analysed is not of concern as the samples were prepared from endophytic extracts. As such their detection confirms the presence of naturally occurring non-pathogenic endo- and epiphytic bacteria in the sample.

Background and Findings

Endophytes: definition, types and source

Endophytes are ubiquitous microorganisms (actinomycetes, bacteria and fungi included), that symbiotically colonize healthy plant tissue, including the cortex, without damaging the plant cells (Lodewyckx et al., 2002; Nair 2014, Teplitski 2009, Rosenblueth 2006, Tyler 2008). They often exist as obligate or facultative microorganisms and their presence causes no harm or disease to the host plant (Lodewyckx et al., 2002; Nair 2014). Plant endophytes commonly exist as mixed colonies of microbial species.

Some bacteria belonging to the genera *Enterobacter* sp. including some coliforms commonly live as plant endophytes (Nair 2014; Taghavi et al. 2009). Bacterial endophytes commonly inhabit the roots, stems, leaves, seed, fruits, and ovules of the host plants, with the most common occurrence in the intercellular spaces and xylem vessels of the plants (Rosenblueth 2006; Nair 2014). The root is the most densely populated location for endophytes and the density decreases progressively from the stem to the leaves (Lamb *et al*., 1996; Quadt-Hallman and Kloepper 1996; Rosenblueth 2006). Endophyte concentrations in plants range between 1×10^3 and 1×10^5 cfu/g plant tissue (Lodewyckx et al. 2002). Endophytic bacteria populations are inconsistent from plant to plant and from species to species, and within the same plant species variations in endophyte populations are influenced by various external factors. Factors that influence the population and density of endophytic bacteria include environmental or external conditions, such as climate, location of the host plant, the genotype and developmental stage of the host, and in some cases the inoculum concentration (Nair 2014; Pillay and Nowak 1997; Tan 2003).

Scientific research is ongoing into the investigation of the mechanisms by which endophytic bacteria enter plants and the identification of their exact location within the plant tissues, as

well as their mode of survival within the tissue. It is thought that bacteria present in the environment, soil or water may enter plant tissues through damaged cell walls or wounds; others also utilize hydrolytic enzymes, such as cellulase and pectinase, to damage the cell walls prior to invading the cells (Zhao 2011).

Advantages of endophytes to plants and subsequently to humans

Of special interest to scientist is the ability of certain plant endophytes including endophytic fungi and bacteria to genetically adapt to their host and develop the ability to biosynthesize some phytochemicals originating from the host plant. Endophytic associations have various advantages to the host plant and indirectly to humans. These microorganisms perform various roles in the host plant including:

- Bio-control agents: some endophytes possess the ability to act as biological agents for the inhibition of plant pathogens (Tyler 2008; Nair 2014). For example, the fungal pathogen *Botrytis cinerea* Pers., responsible for the rotting of tomatoes during storage, is effectively inhibited by the endophytic bacteria *Bacillus subtilis*, isolated from the herb *Speranskia tuberculata* (Bge.) Baill in *in vitro* studies (Wang 2009).
- Biotechnological source of novel and bioactive substances: the ability of some endophytes to synthesize bioactive compounds (e.g. hormones) similar to those produced by the plant for their protection and defense against pathogens has been observed (Nair 2014; Rosenblueth 2006; Tyler 2008). Various novel compounds including the cancer treatment drug Taxol have been isolated from endophytic microorganisms in commercial quantities *in vitro* (Nair 2014).
- Phytostimulation: endophytes assist in the absorption of essential chemical plant nutrients that plants obtain from their environment including carbon, nitrogen and phosphates (Nair 2014). Endophytes also produce beneficial phytohormones like gibberellic acids and cytokinins essential for plant growth and development (Nair 2014; Zhao 2011; Rosenblueth 2006; Tyler 2008).
- Enzyme production: endophytic microorganisms such as some fungi belonging to the genera *Acremonium* (*Acremonium terricola*) and *Aspergillus* (e.g. *Aspergillus japonicas*) have the ability to produce useful commercial enzymes such as cellulases (Nair 2014). Growth-promoting bacterial endophytes have the ability to produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase required to decrease the concentration of ethylene in plants to facilitate plant growth (Nair 2014; Glick 2014; Tyler 2008).
- Antimicrobial activity: some endophytes produce inhibitory compounds that exhibit antimicrobial activity effective against the growth of some species of coliform bacteria including *Bacillus subtilis, Staphylococcus aureus*, and *Escherichia coli* (Nair 2014; Tyler 2008).
- Production of pigments: pigments produced by some endophytic fungi such as *Monodictys castaneae* is capable of impeding the growth of human pathogenic bacteria such as *Staphylococcus aureus, Klebsiella pneumonia, Salmonella typhi,* and *Vibrio cholera*; this pigment has been demonstrated to be more active than streptomycin (Visalakchi 2009).
- Plant nutrient cycling: endophytes have been shown to possess the ability to decompose organic matter, thus maintaining the balance of existing plant nutrients and biomass in the ecosystem (Nair 2014). Some endophytes have been shown to facilitate the biodegradation of the litter of the host plant through their interactions with the saprophytic microbes (Nair 2014).
- Mutual associations between the above and below soil level communities: investigators have observed the ability of some plants harboring endophytes to cause changes in soil conditions or compositions (Nair 2014).

What are pathogens?

Pathogens are microorganisms including bacteria, viruses and protozoa that infect a host (humans and animals) and cause disease in the host (Baylis 2011; National Health and Medical Research Council (NHMRC) 2003). Numerous pathogens exist in the environment and it is impossible to enumerate them all when assessing a water or food sample. Pathogens can be introduced into the food system via irrigation water or other aqueous solutions used in agricultural processes (Pachepsky 2014). Hence, microbial water quality criteria are often adopted by regulatory agencies and water quality standards are enforced by law (Pachepsky 2014).

The United States (US) Environmental Protection Agency (USEPA) in February, 2013 published a Revised Total Coliform Rule (RTCR) in the Federal Register (FR) (78 FR 10269)[1]. This document is a revision of the often cited 1989 Total Coliform Rule (TCR), which seeks to safeguard public health by ensuring the integrity of the nation's drinking water supply, by legislating the monitoring for the presence of microbial contamination including setting legal limits for pathogens including *E. coli* in water supplies.

Similarly, the European Union regulates the quality of drinking water for human consumption under its Council Directive 98/83/EC, which specifies detection limits for *E. coli, Enterococci* and total coliforms.[2] The Second Edition of the World Health Organization (WHO) Guidelines for Drinking-water Quality recommends the assessment of *E. coli* and total coliforms in the evaluation of microbial water quality (WHO, 1993). However, the inadequacy of evaluating total coliforms as an indicator of fecal pollution was discussed in Volume 2 of the Second Edition and the use of alternative indicators e.g. *Enterococci* proposed (WHO, 1996).

Coliforms: definition, types and source

Coliform bacteria are a diverse group of gram-negative, oxidase-negative, rod-shaped functionally–related bacteria belonging to the taxonomic family (Enterobacteriaceae), capable of producing ß-galactosidase enzyme to ferment lactose at 36 ± 2 °C to produce acid within 24 to 48 hours (Ashbolt 2001; Baylis 2011; Health Canada 2012; National Health and Medical Research Council (NHMRC) 2003; Rompre 2002). Bacteria belonging to the Enterobacteriaceae family are gram-negative non-spore-forming facultative anaerobes usually 1-5 µm long and excluding *Saccharobacter fermentans* and some strains of *Yersinia* and *Erwinia* they are capable of reducing nitrate to nitrite (Baylis 2011). Some genera in the Enterobacteriaceae family, like *Salmonella* and *Shigella*, are not considered coliforms (National Health and Medical Research Council (NHMRC) 2003).The family, genera and species of some common coliforms are listed in Table 1 (National Health and Medical Research Council (NHMRC) 2003). Baylis *et al.* (2011) have compiled a list of the genera, species and sub-species of microorganisms belonging to the family Enterobacteriaceae and summarized the common uses of members of the Enterobacteriaceae family and *E. coli* as indicators in various food processing and production applications (Baylis 2011). Coliform bacteria are often normal inhabitants of soil and water milieu that have not been polluted with fecal matter (National Health and Medical Research Council (NHMRC) 2003).

Various molecular methods for the detection of microbial indicator microorganisms and pathogens have been developed including DNA microarray technology, polymerase chain reaction (pcr) methods and fluorescent in situ hybridization (FISH) method (National Health and Medical Research Council (NHMRC) 2003; Rompre 2002). Enzyme-based rapid (results obtained in less than an hour) enumeration methods that detect coliforms not easily detected using conventional methods (selective media) have also been used in water quality sampling (George 2000).

[1] http://water.epa.gov/lawsregs/rulesregs/sdwa/tcr/regulation_revisions.cfm
[2] http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex:31998L0083

Coliform bacteria are generally grouped into three different groups: total coliform, fecal coliform and *E. coli*, a brief description of each group is provided below:

1. Total coliform

Total coliform bacteria are gram-negative, non-spore forming, rod-shaped facultative anaerobes that ferment lactose to produce gas and acid in 48 hours at 35 °C, possess ß-galactosidase enzyme, are commonly found in the environment (e.g. soil, water and food) and are often harmless (Health Canada 2012; Rompre 2002). They belong to the family Enterobacteriaceae.

Due to technological limitations for routine analysis for differentiating *E. coli* from total coliform in the early 1900s, the ease of isolating coliform bacteria from human fecal matter and polluted water, and because *E. coli* is predominantly the coliform bacteria isolated from fecal matter, scientists in the past used total coliform assessments as a reflection of *E. coli* in a sample (National Health and Medical Research Council (NHMRC) 2003). However, the well-known and specific rapid (48 hour) test for thermotolerant (fecal) coliforms was accepted in 1948 (National Health and Medical Research Council (NHMRC) 2003). Despite the development of this widely used method for *E. coli* detection, the use of the total coliform test had become a common substitute for *E. coli* detection. Overtime both tests have remained co-indicators (National Health and Medical Research Council (NHMRC) 2003). The presence of *E. coli* is a widely accepted appropriate specific indicator of fecal pollution, but the use of total coliforms is still regarded as unspecific or probable and not a certain indication of fecal pollution (National Health and Medical Research Council (NHMRC) 2003). Hence, information gained from total coliform measurements can be misinterpreted.

An increase in the identified environmental bacteria, have resulted in changes in the definitions of total coliforms and inferences made from their use as indicator organism. The Australian Drinking Water Guidelines recommends that (National Health and Medical Research Council (NHMRC) 2003):

> ‘Detection of coliform bacteria in the absence of thermotolerant coliforms (or *E. coli*) may be tolerated providing it can be shown that the organisms do not indicate fecal contamination’;

and

> ‘Most coliforms including the thermotolerant coliforms (or alternatively *E. coli*) are not pathogenic but are used as indicators of the possible presence of fecal contamination and enteric pathogens. However, there are many environmental coliforms that are not of fecal origin and are of lesser significance (Fact Sheet 4 – Coliforms)’ (National Health and Medical Research Council (NHMRC) 2003).

Three reasons proposed for regarding total coliforms as an unreliable indicator of the possibility of a waterborne health risk include the ability of coliforms to (National Health and Medical Research Council (NHMRC) 2003):

- Thrive and persist in drinking water distribution systems;
- Be normal inhabitants of soil, water and plants; and
- Not always be present during waterborne disease outbreaks.

2. Thermotolerant coliforms (fecal coliforms)

These coliforms are also known as fecal coliforms due to their role as fecal indicators; some originate from the feces of warm-blooded animals, and they produce acid and gas from lactose at 44.5 ± 0.2 °C within 24 ± 2 h (Ashbolt 2001; Geldreich 1970; Rompre 2002). Some members of this group, predominantly members of the genus *Klebsiella,* do not originate

from feces but originate from different sources including textile, paper and pulp mill wastes (Gauthier 2001). To ensure food safety the fecal coliform test is generally performed to evaluate the possible presence of pathogens of fecal origin in food substances (Baylis 2011; Leclercq et al., 2002).

In a comparison between the commonly used 24-h standardized violet red bile lactose agar (VRBL) method for the enumeration of fecal coliforms to the fecal coliform agar (FCA) method, Leclercq et al. (2002) observed equal sensitivities and specificities for both enumeration methods, except for the detection of lactose-positive non-fecal coliforms such as *Hafnia alvei*, with the potential to form colonies on both FCA and VRBL media (Leclercq et al., 2002). Hence, there is a possibility of obtaining false positive results for fecal coliforms in some instances, as a result of the detection of colonies of confirmed non-fecal coliforms. Both assays were equally sensitive for the detection of *E.coli*, an indication that the enumeration of *E.coli* rather than fecal coliform enumeration is a better indication of the potential of fecal contamination in foods (Leclercq et al., 2002). Doyle and Erickson (2006) suggest the performance of *E.coli* confirmatory tests to decrease the possibility of obtaining false-positive results from fecal coliform tests, which may suggest the presence of bacteria of fecal origin when truly the test detected bacteria commonly associated with plants.

E.coli is considered the best indicator of fecal contamination of foods because this bacteria unlike others in the Enterobacteriaceae family is not persistently found free living in the environment but only exist transiently in the environment; it predominantly originates from the gastrointestinal tract of warm blooded animals and is passed out via feces into the environment (Österblad et al., 1999). Other Enterobacteriaceae are widespread in nature and are considered part of the flora associated with vegetables (Österblad et al., 1999; Wright et al., 1976).

3. Escherichia coli (E. coli)

In 1885, Escherich discovered two kinds of organisms in feces: *Bacterium coli-commune* (*B. coli*) (now known as *Escherichia coli*) and a bacteria similar to Hueppe's Milchsaure-bacterium (currently *Escherichia coli* var*. acidilactici*), which he named *Bakterium lactis aerogenes* on discovery*,* and currently known as *Enterobacter* (formerly *Aerobacter*) *aerogenes* (Hendricks 1978; National Health and Medical Research Council (NHMRC) 2003). The discovery of *Escherichia coli* in fecal matter led to the acceptance of the idea that the presence of these bacteria in water signified fecal pollution of the water (National Health and Medical Research Council (NHMRC) 2003).

Escherichia coli (*E. coli*) are thermophilic coliforms that produce indole from tryptophan and in some cases capable of producing ß-galactosidase enzyme (Ashbolt 2001). *E. coli* is sometimes considered a subgroup of the fecal coliforms groups because it is the most abundant coliform bacteria found in the feces of warm- blooded animals and, therefore, is considered an appropriate group of coliforms to indicate fecal pollution.

The World Health Organization (WHO) proposed the use of three terms to eliminate the ambiguity in the definition of the term 'microbial indicator' as follows (Ashbolt 2001):

- General (process) microbial indicators: These organisms demonstrate the effectiveness of a process e.g. the use of total heterotrophic bacteria or total coliforms for chlorine disinfection (Buchanan 2012);
- Fecal indicator: organisms that show the presence of fecal contamination and only suggest the presence of pathogens e.g. *E. coli*; and
- Index and model organisms: microorganisms that indicate the presence of pathogens and pathogenic characteristics, e.g. *E. coli* used as an index for *Salmonella* and F-RNA coliphages used to indicate human enteric viruses (Buchanan 2012).

Indicator organisms and their uses

Despite the difficulty in isolating and identifying viruses, analysis of human feces and sewage samples have been shown to contain over 100 types of enteric viruses (Payment 1993). Hence the need to adopt practical ways to assess the presence of microorganisms, that could indicate the presence of pathogens in various samples. Major international regulatory authorities include the assessment of bacterial indicators in the evaluation of the microbial food and water quality and regulatory compliance including monitoring and reporting (Pachepsky 2014). However, in recent times the use of total coliform or indicator organism assessments as an indication of fecal pollution and/or pathogenic risk, is thought to be inadequate and subject to controversy (Hendricks 1978; Wu 2011).

The rationale behind the use of the presence or absence of total coliforms in making microbial water or food quality decisions is because these microorganisms normally inhabit the guts of humans and other warm-blooded animals and are transmitted into water together with feces (Hendricks 1978; National Health and Medical Research Council (NHMRC) 2003). The total coliforms group is nonspecific because it grows in ubiquitous environments including the soil and water, for this reason it is persistent and can be detected in water contaminated with fecal matter even after extensive dilution (National Health and Medical Research Council (NHMRC) 2003). Very few coliforms are considered to be health risks, and their presence only indicates that there is a potential for fecal pollution of a water sample, which could indicate the possibility of pathogens being present in the sample (Craun 1997; Hendricks 1978; National Health and Medical Research Council (NHMRC) 2003). Numerous studies provide conflicting results on quantitative relationships between indicators and pathogens. Therefore, positive coliform results do not confirm that pathogens are present in that sample.

The bacteria *Escherichia coli* is the most abundant microorganism in the total coliform group originating from mammalian feces, and is considered the most specific indicator of fecal pollution, because it rarely grows in the environment, see Table 2 (National Health and Medical Research Council (NHMRC) 2003). For this reason the absence or presence of *E. coli* in a sample is considered as an indication of the probable absence or presence of microbial fecal pollution including bacteria, viruses and protozoa in a water sample (National Health and Medical Research Council (NHMRC) 2003).

Indicator-pathogen relationship

An indicator microorganism's ability to multiply in an environment, variations in their environmental resistance relative to their in situ behavior, the removal and destruction rates of the indicator relative to its target pathogen, all influence the validity of using the indicator for that target organism (Ashbolt 2001).

Despite the recognized usefulness of indicator organisms in assessing public health risk associated with water source or type, their effectiveness to indicate pathogenic risk is confounded and often questioned by scientists (Ashbolt 2001; Wu 2011). Wu *et al*. (2011), searched literature published from 1970-2009 and obtained data on 540 cases of independent indicator microorganism and pathogen correlations, to assess factors influencing this correlation they conducted a logistic regression analysis (Wu 2011). Factors evaluated included indicator groups, pathogen groups, pathogen sources, water source/types, and sample size (Wu 2011). Others were quantity of pathogenic samples, detection methods, publication year and statistical methods (Wu 2011). Of these factors sample size and the quantity of samples testing positive for pathogens were most important in the evaluation of the correlation. On the contrary pathogen sources, detection method and the other factors had a minimal effect on the observed correlation (Wu 2011). Results of the quantitative relationships between indicator organisms and pathogens are often conflicted, as such the assessment of health risk based on results from indicator organism assessments can be challenging. As such the absence of an indicator organism in a water

sample does not imply the absence of pathogenic microbes; neither does their presence imply a public health risk (Wu 2011).

Various investigators have attempted to identify correlations between indicators and pathogens, as a means of identifying the most appropriate indicators that show the existence of pathogens (Carter 1987; Noble 2001; Pachepsky 2014; Payment 1993; Stetler 1984). Although the results are mixed, some observed significant correlations (Payment 1993; Stetler 1984), while others observed no correlations between indicator microorganisms and the presence of pathogens (Ashbolt 2001; Carter 1987; Harwood 2005; Noble 2001).

Alternatives to using *E. coli* such as enterococci as a coliform bacteria indicator for the detection of fecal pollution have been proposed (Pinto 1999; Yates 2007). An analysis of fecal streptococci isolates obtained from a variety of environmental samples showed that 84.4% were biochemically confirmed fecal species (Pinto 1999).

Endophyte–coliform (including fecal coliform) relationship:

Enteric bacteria including members of the Enterobacteriaceae family, such as *Salmonella* and *E. coli* are common inhabitants of the plant cortex. The discovery of enteric bacteria acting as endophytes in plant hosts coupled with the association of foodborne outbreaks due to the consumption of raw produce has led scientists to believe that these bacteria live as endophyte in the host plant. In particular *Salmonella* has been observed to live endophytically in seeds of tomato (Guo 2001). Some nonpathogenic Enterobacteriaceae bacteria have been isolated from edible plants including dandelion, tomatoes and potatoes; many of the isolates were observed to be antibiotic resistant and had the affinity to adhere to human erythrocytes (Tyler 2008). *Enterobacter* sp. have been described as plant growth promoters (Lodewyckx et al., 2002; Rodriguez and Fraga, 1999; Taghavi et al., 2009). Some endophytes are also seed borne and can be inherited from generation to generation of plants (Cooley 2003; Nair 2014 and Wang 2006).

In several experimental studies *E.coli* O157:H7 has been shown to colonize the cells of lettuce (Bernstein 2007; Franz 2005; Solomon 2002). Tissue analysis of surface-sterilized lettuce revealed 3.95 log cfu/g of *E.coli* O157:H7 (Franz 2005). Similarly 2.57 log cfu/g *Salmonella enterica* serovar Typhimurium, has been detected on surface-sterilized lettuce (Franz 2005). In tomato *Salmonella Montevideo* was the more persistent endophyte, whereas *Salmonella Poona* was more prevalent (Guo 2001). However, not all strains of *Salmonella* are capable of endophytically colonizing plant tissues. These studies demonstrate the potential for pathogenic bacteria from external sources, such as fecal effluent, to become embedded in some plants and live as endophytes given the right conditions for growth and survival. Hence a substance that tests negative for fecal coliforms including *E.coli* can be presumed to be devoid of external fecal contamination.

Enterobacteriaceae are detected using fecal coliforms assay, but Enterobacteriaceae other than *Escherichia coli* are often associated with plants and as such are not suitable indicators of true fecal contamination (Doyle and Erickson, 2006; Edberg et al., 2000).

Brief Summary of Analytical Results (5-Batch Analysis)

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identification of the bacteria present in the samples indicated the presence of three plant associated coliform bacteria, namely: *Enterobacter cloacae*, *Enterobacter hormaechei* and *Pantoea* sp. All three bacteria species are non-pathogenic strains of the Enterobacteriaceae family that have been identified as common plant endophytes or epiphytes. More so the low levels (1×10^3 CFU/mL) of these three bacteria detected in the samples, mitigates concern for a microbial hazard risk. Furthermore the results demonstrate the lack of fecal contamination as indicated by the lack of *E. coli* in the samples tested (see Table 3 and 4). A brief background of the three bacteria detected is presented below:

- [redacted] is an endophytic symbiont that is widely spread in nature and mainly inhabits the rhizosphere of plants (Hinton and Bacon, 1995). It is also isolated from stems and leaves and as such is also a phyllosphere colonizer (Ladha et al., 1983). They are nitrogen-fixing bacteria (Raju et al. 1972, Zhu *et al.*, 1986), with the potential to act as biocontrol agents of various diseases in turf, corn and vegetables (Hinton and Bacon, 1995). Tirosh and Glick (2001) demonstrated that *Enterobacter cloacae* CAL3 was a growth promoter for tomato plants. Although some strains have been known to cause nosocomial infections in immunocompromised patients, none pose a significant human health risk to the general public. Furthermore, it has been demonstrated that isolates of *Enterobacter cloacae* from drinking water sources differed from those found in hospital environments and/or isolated from patients, implying that isolates from water sources were not constituting a public health risk (NHMRC p.15, 2003).
- [redacted] is a bacterium found in the soil, plants and water. It has been shown to exhibit growth stimulating effects and the potential to grow in saline conditions (Egamberdieva et al., 2008). *Enterobacter hormaechei* is also described as an enhancer in the phytoremediation of polluted environments (Chen et al., 2012).
- [redacted] is an epiphyte, hence it is commonly found on the surface of plant leaves (Sabaratnam and Beattie, 2003). This bacterium served as a model organism in various studies about phyllosphere ecology (Leveau and Lindow, 2001). *Pantoea agglomerans* is used for the biological control of several diseases in pome fruits (Braun-Kiewnick et al., 2012). The efficacy of various strains belonging to the genus *Pantoea* to control the fire blight bacterium *Erwinia amylovora* has been demonstrated (Johnson et al., 2004; Pusey, 2002; Stockwell et al., 2010).

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Recommendations and Conclusions

Based on our review of the scientific literature, ENVIRON has concluded that the detection of total coliforms only indicates that there is some potential for the presence of pathogens in a sample. Some plant endophytes including some strains of *Salmonella* and *E. coli* can be pathogenic coliforms. A positive coliform result does not confirm the presence of pathogens in a sample, unless a specific pathogen has been detected in the sample. Conversely, the detection of fecal coliforms in a sample is a much stronger indicator that pathogenic coliforms could be present in that sample than a total coliform result. Because endophytic bacteria can include a mix of benign or non-pathogenic bacteria and coliform bacteria, the detection of total and/or fecal coliform bacteria in an isolate prepared from plant endophytes is not positively indicative of external bacterial contamination, but confirmatory of the presence of the endophytes in the sample. However, the endophytic behavior of certain bacteria, especially pathogenic fecal coliform bacteria such as *E.coli* O157:H7 is of food safety concern, because this could render sanitization and antimicrobial treatments ineffective in ensuring that horticultural products are devoid of such bacteria, and could potentially lead to food-borne illness even after such precautionary measures are taken.

The data in Table 2 demonstrate that *E. coli* constitutes more than 92 % of total coliform results in human and animal feces. Validating the presence or absence of specific pathogenic organisms in a sample requires testing for specific known pathogens that may or may not belong to the coliform group. The process of performing confirmatory tests for

currently known pathogens associated with a particular sample is likely to be impractical and time consuming. Therefore, it is appropriate to assess key indicator microorganisms such as *E. coli* as an indication of possible pollution or microbial pathogenic contamination of a sample. Because *E. coli* was not detected in any of the samples of the five batches of samples analysed on both occasions and the coliforms that were found were determined and identified as [redacted] all of which have all been described as plant growth promoting endo- and epiphytic bacteria, it can be concluded that the sample is not pathogenic but consist of naturally occurring plant associated coliform bacteria.

10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

Strengths and Limitations; Approach and Reliance

ENVIRON's literature review included published peer reviewed literature (e.g. from PubMed, Google Scholar, American Society of Microbiology (ASM)), publications by international regulatory organizations (e.g. World Health Organization (WHO)) and information from the websites of authoritative bodies and governmental entities. This review has been prepared exclusively for use by De Ceuster N.V., and such other persons or entities whose reliance is explicitly authorized in writing by ENVIRON. The conclusions presented in this memorandum represent ENVIRON's best professional judgment based upon the information synthesized from the literature reviewed and cited as of the date of the review. It also included information provided by De Ceuster N.V. This review is not intended as legal advice. ENVIRON makes no representations or warranties, express or implied, about the subject product.

Closing

We trust that we have addressed fully herein your questions; however, if you have any additional questions or comments, please contact us.

Table 1: Family, Genera and Species of Some Common Coliforms

Family	Genera	Species
Enterobacteriaceae	Escherichia	*Escherichia coli* (*E. coli*)
	Klebsiella	*Klebsiella pneumonia* (*K. pneumoniae*)
	Enterobacter	*Enterobacter amnigenus* (*E. amnigenus*)
	Citrobacter	*Citrobacter freundii* (*C. freundii*)

Source: National Health and Medical Research Council (NHMRC). Review of coliforms as microbial indicators of drinking water quality. 2003. Australian Government; NHMRC; http://www.nhmrc.gov.au. Investing in Australia's Health.

Table 2: Distribution of Some Common Coliform Genera in Human and Animal Feces(1)

		% of Total Coliforms in Samples		
Family	**Species**	**Human feces**	**Animal feces**	**Reference**
Enterobacteriaceae	*Escherichia coli* (*E. coli*)	96.8	94.0	Dufour (1977)
		94.1	92.6	Allen and Edberg (1995)
	Klebsiella sp.	1.5	2.0	Dufour (1977)
	Enterobacter/*Citrobacter* sp.	1.7	4.0	Dufour (1977)
		5.9	7.4	Allen and Edberg (1995)

Source: National Health and Medical Research Council (NHMRC). Review of coliforms as microbial indicators of drinking water quality. 2003. Australian Government; NHMRC; http://www.nhmrc.gov.au. Investing in Australia's Health.

Notes: (1) = Once feces leaves the body and makes its way down the sewer, the proportions of coliforms that are *E. coli* drops to about 30% as the other coliforms start to grow (Geldreich, 1978).

Abbreviations:
sp. = specie(s)

10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

Table 3: Microbial analysis of five different batches of *Pepino mosaic* virus, strain CH2, isolate 1906 conducted on 08/08/2014 (Analysis: SGS Belgium NV; Stichelbaut, 2014)

Microorganism	**Batch 1:** [redacted]	**Batch 2:** [redacted]	**Batch 3** [redacted]	**Batch 4** [redacted]	**Batch 5** [redacted]	**Method**
Salmonella (37°C)/25 mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	AFNOR BRD-07/6-07/04
Vibrio sp. (37°C)/25 mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO/TS 21872-2 modified
Shigella (42°C)/25 mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO 21567:2002 modified
Bacillus cereus (30°C)/mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO 7932
Coliforms (30°C)/mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO 4832
E.coli (37°C)/ 1 mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO/TS 16649-3
Yeasts (25°C)/mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO/TS 21527-1
Moulds (25°C)/mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO/TS 21527-1
Staphylococcus aureus (37°C)/ 1 mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO 6888-3
Total Aerobe Count (30°C)/mL	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	ISO 4833

Source: SGS Analytical Report: AN14-20299 provided by De Ceuster N.V.

Abbreviations:
mL = milliliter; °C = degrees Celsius; SGS = Société Générale de Surveillance; ISO = International Organization for Standardization; spp = species; ISO/TS = International Organization for Standardization Technical Specifications; AFNOR BRD = AFNOR group standards

10.1.c Wob juncto 63.2.a Vo 1107/2009 juncto 39.2.a Vo 178/2002

Table 4: Total coliform and fecal coliform analysis of five different batches of *Pepino mosaic* virus, strain CH2, isolate 1906 conducted on 28/01/2015 (Analysis: SGS Belgium NV; [redacted]

Batch Number	***E. coli* /mL (44°C)/mL**	**Coliforms (30°C)/mL**	**Fecal coliforms (44°C)/mL**
1	[redacted]	[redacted]	[redacted]
2	[redacted]	[redacted]	[redacted]
3	[redacted]	[redacted]	[redacted]
4	[redacted]	[redacted]	[redacted]
5	[redacted]	[redacted]	[redacted]
Method	ISO 16649-2	ISO 4832	NV V08-060

Source: SGS Analytical Report: AN15-02366 provided by De Ceuster N.V.

Abbreviations:
mL = milliliter; °C = degrees Celsius; SGS = Société Générale de Surveillance; ISO = International Organization for Standardization

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Attachment 1

Glossary of key fecal indicator microorganisms (Adapted from: WHO 2001: Indicators of microbial water quality)

- **Fecal streptococci (FS)**: comprise of gram-positive, catalase-negative cocci from selective media (e.g. azide dextrose broth or m Enterococcus agar), that possess the Lancefield group D antigen, grow on bile aesculin agar at 45°C and belong to the genera *Enterococcus* and *Streptococcus.*
- **Enterococci**: consist of fecal streptococci majority of which are members of the genus *Enterococcus* and grow at pH 9.6, in 6.5% sodium chloride (NaCl), at 10° and 45°C with the ability to reduce 0.1% methylene blue and show resistance to 60°C for 30 min. These micro-organisms also grow aerobically at 44±0.5°C and hydrolyze 4-methlumbelliferyl-β-D-glucoside (MUD, detecting β-glucosidase activity by blue florescence at 366 nm), in the presence of thallium acetate, nalidixic acid and 2,3,5-triphenyltetrazolium chloride (TTC, which is reduced to the red formazan) in the specified medium (ISO/FDIS 7899-1 1998).
- **Sulphite-reducing clostridia (SRC)**: Gram-positive, spore-forming, non-motile, strictly anaerobic rods that reduce sulphite to hydrogen sulphide (H_2S).
- ***Clostridium perfringens:*** subgroup of the sulphite-reducing clostridia that reduce nitrate, produce lecithinase and acid phosphatase, hydrolyze gelatin, ferment lactose, sucrose and inositol to produce gas, and ferment milk to make a stormy clot.
- **Bifidobacteria**: pleomorphic (capable of producing branching bulbs (bifids), clubs, coccoid, coryneform, Y and V forms), gram-positive bacilli, catalase-negative, non-acid-fast, non-spore-forming, non-motile, obligate anaerobes and all lactose fermenting with the exclusion of these three insect species; *B. asteroides, B. indicum* and *B. coryneforme.*
- **Bacteriophages (phages)**: ubiquitous environmental bacterial viruses e.g. somatic coliphages, male-specific RNA coliphages (F-RNA coliphages), often used for water quality testing and to model human enteric viruses.
- **Coliphages**: “Somatic coliphages attack *E. coli* strains via the cell wall and include spherical phages of the family *Microviridae* and various tailed phages in 3 families. The F-RNA coliphages attack *E. coli* strains via the sex pili (F factor) and are single-stranded RNA non-tailed phages in four groups” (Ashbolt 2001).
- ***Bacteroides fragilis* bacteriophages**: “These infect one of the most abundant bacteria in the gut, belong to the family Siphoviridae with flexible tail (dsDNA, long non-contractile tails, capsids up to 60 nm). Phages to the host strain, B. fragilis HSP40 are considered to be human-specific, but phages to B. fragilis RYC2056 are more numerous and not human-specific (Puig et al. 1999)” (Ashbolt 2001).