Prevention and Control of Microbial Growth in Water-based Crop Protection Formulations
Picture Front Cover:
Bacillus species (vegetative stage and endospore formation)
Magnification: x 2,200; (Scanning Electron Microscopy)¹

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Prevention and Control of Microbial Growth in Water-based Crop Protection Formulations
Acknowledgments

The MSCSG would like to express their thanks to the authors:
Dipl.-Ing. Heiko Wolf, Dr. Bruce E. Urtz, Dr. Marten Snel and Dr. Wolfgang Schäfer
Dear Reader,

Our entire Crop Protection industry is strongly committed to Responsible Care™. Therefore, we will where appropriate provide up to date information, training, and support ongoing improvements to the quality of our operations and products.

Contamination of crop protection products can be caused by active ingredients or intermediates of products as well as other extraneous chemicals, which were previously present in the equipment, but also by microorganisms like bacteria, yeasts and molds. An increasing number of aqueous formulations in the product mixes of all manufacturers of crop protection products require extra attention during the manufacturing process with regards to “Microbial Growth Prevention” management.

This is the reason why a team of experts of the member companies of the European Crop Protection Association (ECPA) have written the booklet “Prevention and Control of Microbial Growth in water-based Crop Protection Formulations”.

The objective of this booklet is to inform all toll manufacturers worldwide on implementation of the best practices provided in this booklet to ideally prevent or if necessary correct microbial growth. This will help reduce the likelihood of errors resulting in microbial contamination and result in continuous improvement of the Quality Standards in our entire industry.

Irrespective of the causes of contamination incidents, the consequences are always the same: additional production costs, delays in delivery, possible disposal of contaminated product causing an unnecessary environmental burden. Not to be underestimated is the danger that these incidents could damage the reputation not only of the company where the incident occurred, but also the reputation of our entire branch.

I cannot stress the importance of good Microbial Growth Prevention management enough and I hope that this booklet will not only serve as basis for in-depth discussions of this topic within your companies, but will result in adoption of the best practices described. This will optimize your chances of preventing Microbial Growth incidents in crop protection products manufacturing facilities.

George L. Poe

Chairman Manufacturing and Supply Chain Steering Group (MSCSG) of the European Crop Protection Association
10. Controlling Microbial Growth in the Production Process through Biocide Addition 22

11. Plant Hygiene 24
   11.1. Areas Susceptible to Microbial Growth 24
   11.2. Testing for Microorganisms in the Production Facility 27

12. Plant Cleaning and Disinfection 28
   12.1. Mechanical Cleaning 28
   12.2. Chemical Cleaning 28
   12.3. Chemical Disinfectants 29
   12.4. Hot Water or Steam 30
   12.5. Treatment Strategy 30
   12.6. Drying 31

13. Rework of Formulations Containing Microorganisms 31

14. Glossary 32

15. Microbial Contamination Prevention Checklist 34
The presence of microorganisms in liquid agrochemical products may or may not be harmful to that product. In situations where it is harmful, the physical properties of the formulation may be affected including pH, viscosity, dispersion stability, storage stability, sedimentation, or even unwanted degradation of the active ingredient. Furthermore, a bad odor and/or gas may develop. These quality issues must be avoided to guarantee full customer satisfaction and to ensure the producer is seen as a reliable partner for agrochemical products.

Bulging containers due to off-gassing from microbial growth
Loss of viscosity, phase separation and sedimentation due to microbial thickener degradation

It is not realistic to expect aseptic conditions in a formulation plant. Rather, the objective is to prevent microbial growth in the final product by maintaining an adequate level of plant hygiene in combination with the proper biocide.
2. **Purpose and Scope**

The purpose of these best practices guidelines is to support formulation sites and manufacturers with measures which should reduce and where possible prevent the risk of microbial growth developing in agrochemical products. After reviewing a number of case histories, an overview of microbiology will be given including description of test methods that can be used to detect the presence of microorganisms. The use of biocides to prevent microbial growth in agrochemical formulations will be discussed. Cleaning and disinfection products and procedures are also described. These measures should keep the formulation plants in good condition.

The scope of the information on best practices is to provide guidelines on how to prevent microbial growth in water-based formulations like suspension concentrates, suspo-emulsions, low concentration soluble liquids and seed treatment formulations. Solid formulations like gels, baits or pellets are not included in the scope of this document. However, most of the principles described here are also applicable to those products as well.

3. **Case Histories**

3.1. A suspension concentrate (SC) developed microbial growth caused by using poor quality water. The water was obtained from a water purification plant that utilized ion exchange tanks. The tanks were not periodically disinfected. Additionally, the water was supplied to the formulation plant in rubber coated tank wagons, sometimes stored under direct sunlight during hot summer days. A plant inspection showed microbiological infestation of the water storage tanks, and parts of the formulation plant with large surface areas such as filter devices and heat exchangers. These parts and others (ball valves, seals between flanges, plastic parts etc.) were not sufficiently cleaned which allowed for the formation of biofilms.

3.2. Microbial growth developed in another SC triggered by the use of microbiologically contaminated rinse water. The rinse water was recycled to reduce disposal of waste water. However, the rinse water tanks were not sufficiently treated, so they served as the perfect environment for microbial growth. Several hundred thousand liters of formulated product needed to be reworked with huge effort.

3.3. Several SC products had microbial growth. As a result, various parts of the formulation plant were inspected for the presence of microorganisms. Parts of the formulation line tested positive for microbial growth. The investigation showed incorrect production practices, bad housekeeping and insufficient disinfection procedures. Non-used parts of the production line were still connected by ball valves and filled with water for months.
Filter devices were still in poor condition even though they had been cleaned! Prior to the investigation an unsuitable testing procedure was used, which always showed negative results (i.e. no microorganisms).

3.4. A formulation plant for soluble liquids (SL) tested positive for the presence of microorganisms after changing to another water purification plant. The water purification plant included several “dead spaces” that could not be cleaned effectively.

3.5. Microbial growth was detected in products from a SC formulation plant. During an investigation, several hot spots were discovered from where microorganisms could have originated. After disinfection of the plant, everything seemed satisfactory. However, after a few weeks the problem returned. After further investigation, biofilms were identified as the root cause. Although a chemical disinfection was performed, it was not completely effective in removing the biofilms.

3.6. To reduce water consumption, product waste, and disposal costs of rinse water, a SC formulation plant decided to collect the first 600 litres of rinse water and store this water. This rinse water had up to 25% product and was stored in intermediate bulk containers (IBC) in a warehouse during the summer. The intention was to reuse this material when the same SC was formulated again in the next campaign. Temperatures in the warehouse were high during the storage period, and the water mixture began to give off an odor. Investigation revealed that the bacteria count was high and the stored rinse water had to be discarded. The plan to reduce rinse water disposal costs was abandoned. *Aqueous rinsates especially when stored over long periods between campaigns are not suitable for recycling into new product.*

3.7. A shipment of SC was inspected by customs inspectors and found to be out of specification due to viscosity loss and phase separation. It was stopped at the border and could not enter the country. It was later determined that the thickener was degrading due to the presence of microbial enzymes. An investigation at the manufacturing site revealed both poor hygiene in the thickener tank, and a poor quality thickener. When modifications were made to the thickener preparation system and the raw material was replaced with a higher quality thickener (lower colony forming unit count [CFU]), the problem was resolved and did not occur again. **Bacteria:** These are small (micron size) usually single cell organisms that reproduce by dividing into two. Various species can be found in raw materials and production plant environments. Some examples include *Bacillus, Burkholderia* and *Pseudomonas.*
4. Microbiology and Agrochemical Products

4.1. Types of Microorganisms
Microorganisms that survive and grow in agrochemical products can be divided into two major groups: bacteria and fungi.

**Bacteria:** These are small (micron size) typically single cell organisms that reproduce by dividing into two. Various species can be found in raw materials as well as in other environments of production plants. Examples include: *Bacillus, Burkholderia* and *Pseudomonas.*

![Pseudomonas species](image1)

*Pseudomonas species*
*Magnification:*
x2, 600; *Scanning Electron Microscope (SEM)*

**Fungi** can be further divided into yeasts and molds.
- **Yeasts** are small (micron size) single cell organisms that reproduce by budding.

![Yeast (single cells)](image2)

*Yeast (single cells)*
*Magnification:*
x1,600; *(SEM)*

- Molds range in size from small (micron size) to large enough structures that can be seen with the naked eye. They grow by producing multicellular “threads” called filaments or hyphae. Sometimes these filaments can form large mats (mycelia) on the surface of water-based formulations. In the reproductive phase, molds form spores which become airborne. This results in spores being freely distributed in the environment making it possible for these fungal spores to enter products, raw materials and manufacturing equipment etc.

![Molds (filamentous growth; hyphae forming spores)](image3)

*Molds (filamentous growth; hyphae forming spores)*
*Magnification:*
x340; *(SEM)*

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4.2. Factors that Affect Microbial Growth

Production plants for water-based formulations are typically operated at temperatures between 20°C and 40°C. This temperature range is optimal for the multiplication of many of the microorganisms commonly found in these facilities. In addition, other conditions needed for microbial growth may be present such as:

- water (even with dry products microbial growth may occur if the relative humidity is high, for bacteria > 90%, for fungi > 60%)
- nutrients (primarily carbon and nitrogen sources)
- favorable pH

The typical pH range for bacterial and fungal growth is shown below.

![pH Chart]

In general, fungi grow better at lower pH values than bacteria. However, there are always exceptions. For example, some fungi can grow above pH 9, and there are some bacteria that can grow at an acidic pH. The pH of most liquid agrochemical formulations fall within a range which can support the growth of both bacteria and fungi.

Microorganisms also have different requirements with regards to oxygen. Some microbes only grow in the presence of oxygen (aerobes). Some microbes only grow in the absence of oxygen (anaerobes), while some microbes can grow with or without oxygen (facultative anaerobes).

When bacteria grow they will typically go through four phases. In the lag phase the bacteria are adapting to their new environment and getting ready for cell growth and division. During the exponential phase the bacteria are reproducing (i.e. dividing) very quickly. Under ideal conditions this can occur in as little time as 20 to 30 minutes. This is why the problems associated with microbial growth can occur very quickly. Eventually the bacteria will run out of nutrients and their growth and reproduction will stop (stationary phase). Over time the bacteria will begin to die off (death phase). If a sample is tested after the death phase, microbial growth may be overlooked as the source of the
problem. The maximum level of microbial growth, and the time it takes to go through the different phases will vary depending on factors such as nutrient availability, temperature and pH.

4.3. Spores
Molds are capable of producing spores that can be readily distributed through the air. Therefore, they tend to be present on almost any surface. If conditions become favorable for growth (moisture, nutrients, etc.), the spores will germinate, and the fungus will start to grow.

Some bacteria (e.g. *Bacillus* sp.) are capable of forming endospores. This is a protective dormant stage, and the spores are resistant to biocides, disinfectants, and unfavorable environmental conditions such as drying. As a result they may get introduced into the manufacturing process via dry products. As with fungal spores, if conditions become favorable, the endospores will germinate and bacterial growth resumes. Even though some biocides may not be very effective in killing endospores, they usually prevent the spores from germinating into growing cells.

*Bacillus species* (vegetative stage and endospore formation)
*Magnification: x 2,200; (SEM)*

1 ©Dennis Kunkel Microscopy, Inc.
4.4. Enzymes
Thickeners like Xanthan gum consist of large polymers of sugar molecules. Some bacteria and fungi are capable of producing enzymes that can break these polymers into the smaller sugar molecules which the microorganisms can use for food. When thickener degradation occurs, the formulation can lose viscosity and separate into phases. The enzymes are produced outside of the bacterial or fungal cells, and can be fairly stable. Therefore, even if a biocide is added to kill the microorganisms, the enzymes may persist and continue to degrade the thickener.

4.5. Biofilms
Biofilms are a common and extremely critical source for microorganisms in water based systems. A biofilm is mostly a slimy layer composed of microorganisms, nutrients, metabolites and water. Often times it will be a part of a deposit that contains the chemical product being made in the process. A biofilm can develop and spread on floors, pipes, vessels, holding tanks and any other surface that becomes wet. All materials typically used in agrochemical plants are at risk irrespective of whether they are made of metals, plastics or rubber parts.

Microorganisms inside a biofilm are much harder to kill and control than microbes outside a biofilm. For example, levels of sodium hypochlorite (e.g. 1-3 ppm as Cl₂) which kill most microbes in water will have little, if any, effect on those same microbes in a biofilm. Furthermore, once a biofilm becomes established it can detach and spread microbes further downstream in the process.
Different microorganisms thrive and grow in different environments, and the same is true for chemical products. First, a microbe has to access the product via water, raw materials, air, etc. Then it must be able to survive and grow in the product. With time the microbes can become highly adapted to that environment, even when the product has a relatively low nutrient level to support microbial growth.

Susceptible to microbial growth are *water based formulations* like:

- **Suspension Concentrates (SC)**
- **Suspo - Emulsions (SE)**
- **Soluble Liquids (SL)**
- **Seed Treatment Formulations (FS)**

Granular, pellet and block baits are also susceptible. However, these formulations fall outside the scope of this document, but also require a proper plant hygiene program.

### 5.1. Suspension Concentrates (SC)

Suspension concentrates (SC) (often referred to as “flowables”) contain the active ingredient(s) as solid particles dispersed in a continuous aqueous phase. Dissolved in the aqueous phase are mainly additives like thickeners and surface active ingredients beside some minor additives. The ingredients in the aqueous phase provide sufficient nutrition for microorganisms.

### 5.2. Suspo - Emulsions (SE)

Suspo - emulsions (SE) have almost the same matrix as SCs, except that an additional, lipid soluble active ingredient is present emulsified in an organic phase besides the aqueous phase with the solid particles.

### 5.3. Soluble Liquids (SL)

Soluble liquids (SL) contain the active ingredient as its salt, which is completely dissolved. Other additives are normally also present. Microbial growth mainly occurs in SLs when the salt content is below 20 % w/w. At high salt concentrations, high osmotic pressure outside the cells of the microorganisms leads to water transfer from inside the cells into the outside liquid phase, and the microbes dehydrate.

### 5.4 Seed Treatment Formulations (FS)

Seed treatment formulations (FS = flowable concentrates for seed treatment) are stable water-based flowable concentrates for seed treatments. FS formulations are often applied in undiluted form. The formulation is very similar to that of SCs, but contains a sticker to help the FS to adhere better to the seeds.
If a formulated product is found to be susceptible to microbial growth, a biocide is commonly added to the formulation. The biocide should not only prevent microbial growth, but also have no effect on the formulation’s performance and toxicological profile.

6.1. Actives
Biocide actives used for the prevention of microbial growth in chemical products can be separated into two major modes of action, the electrophilic and the membrane active substances.

<table>
<thead>
<tr>
<th>Electrophilic Substances</th>
<th>Membrane Active Substances</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aldehydes</strong></td>
<td><strong>Alcohols</strong></td>
</tr>
<tr>
<td>- Formaldehyde</td>
<td>- Benzalcohols</td>
</tr>
<tr>
<td>- Formaldehyde releasing compounds</td>
<td>- Phenoxyalcohols</td>
</tr>
<tr>
<td>- Glutaraldehyde</td>
<td></td>
</tr>
<tr>
<td>- Glyoxal</td>
<td></td>
</tr>
<tr>
<td><strong>Activated Halogen Compounds</strong></td>
<td><strong>Weakly Lipophilic Acids</strong></td>
</tr>
<tr>
<td>- Bronopol</td>
<td>- Benzoic acid</td>
</tr>
<tr>
<td>- Chloracetamide</td>
<td>- Sorbic acid</td>
</tr>
<tr>
<td>- Dibromodicyanobutane</td>
<td></td>
</tr>
<tr>
<td>- Dibromonitrilopropionamide</td>
<td></td>
</tr>
<tr>
<td><strong>Isothiazolinones</strong></td>
<td><strong>Cation Active Substances</strong></td>
</tr>
<tr>
<td>- Methylisothiazolinone (MIT)</td>
<td>- Quarternary Ammonia compounds</td>
</tr>
<tr>
<td>- Chloromethylisothiazolinone (CMIT)</td>
<td>- Guanidine</td>
</tr>
<tr>
<td>- Benzisothiazolinone (BIT)</td>
<td></td>
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</tbody>
</table>

Formaldehyde and formaldehyde releasing compounds have been used extensively in the past. However, regulatory constraints now preclude their use in agrochemical products in some regions.

The biocide actives that can be used for preventing microbial growth in agrochemical products is a small subset of the list above, and will vary depending on the country or region. The most commonly used actives are the isothiazolinones. Some biocide products may have a single active (e.g. BIT), others may contain a combination of actives (e.g. CMIT and MIT).
6.2. Efficacy Testing
Biocide challenge studies are commonly used to select which biocide is most effective in preventing microbial growth. Typically, aliquots of the formulated product are dosed with different concentrations of the biocides which are being evaluated. Then the product samples are challenged with a mixture of microorganisms capable of growing in the product, if no biocide would be present. Ideally, these microbes should come from the product itself and/or from the manufacturing site(s) where the product will be made. If there is some product available that already has microbes present, this can also be used as part of the challenge. Usually, this is the best type of challenge inoculum, because the microbes are already conditioned to growing in the product.

After the challenge, the product samples are tested for the presence/absence of microbes (section 7). Usually several challenges are conducted over a period of time (e.g. 1 - 2 months). After each challenge, the samples are tested for microbial growth.

6.3. Stability
Biocide stability must be considered when choosing a biocide. At the very least, a biocide should control the microorganisms present at the time of product manufacturing. The length of time that a product will be protected against further microbial growth will depend on which microbial pressures the product will be exposed to, as well as the stability of the biocide. Biocide stability can be affected by a variety of factors including product chemistry, high levels of amines, reducing agents, pH and storage temperature.
6.4. Bio-availability
Another important aspect is the bio-availability of biocides. For example, benzisothiazolinone (BIT) and methylisothiazolinone (MIT) are sensitive to polar molecules. They may adsorb at the surface of such particles (e.g. dispersed active ingredients or even fillers like highly porous silica derivates) and will thus no longer be available as a biocide.

The model diagram below shows how a biocide concentration may change over time. After a biocide is added, a portion may be adsorbed or interact with the formulation matrix; another part might be used up in killing existing microorganisms in the product. Only the residual amount is available for the long term preservation of the product. This initial loss is important to consider in product development.

6.5. Tolerance
Microorganisms can not only adapt to growing in various chemical products, they can also adapt to the biocide being used to control them. This is referred to as biocide tolerance. As this develops a concentration of biocide that was once effective may no longer be effective in controlling the microorganisms. This is often a problem when Pseudomonas bacteria are present, and the biocide contains only one active ingredient. If the biocide contains multiple actives in sufficient concentration, biocide tolerance is much less likely to occur.
Various methods can be used to test for the presence of microbial growth in a chemical product. When choosing a method, one must consider various factors including whether or not rapid results are needed. Also, how precise does the testing need to be? Do we just need to know presence or absence, or do we need something more quantitative? Both the skill level of the person(s) performing the tests and the availability of suitable laboratory equipment are also important considerations.

7.1. Standard Plate Count Method
The most common way to test for the presence of bacteria or fungi is to spread an aliquot (0.1 ml) of the sample onto an agar plate. If bacteria or fungi are present, they will grow on the agar plate, and after a suitable incubation period will form colonies that can be counted. Different types of agar plates are available for growing different types of bacteria and fungi. For testing chemical products, usually non-selective media are chosen capable of growing a broad range of microorganisms (e.g. Plate Count Agar, Tryptic Soy Agar, etc.). Typical incubation conditions for bacteria are 48 - 72 h at 30°C, for fungi 48 - 120 h at 25°C.

If the concentration of bacteria and/or fungi is high, and an accurate count is necessary then the sample must be diluted. Typically a series of 1:10 dilutions are made up in sterile buffer and each dilution is spread onto the agar plate. After the incubation, any agar plate that has a colony number between 30 - 300 is counted. The number of colony counts is multiplied by the dilution factor to determine the concentration of microorganisms in the original sample. This is reported as colony forming units (CFU) per ml or g of sample.

If only qualitative or semi quantitative results are required, a sterile swab can be inserted into the sample and then swabbed onto an agar plate. Based on the number of colonies that are detected, one can rate the level of microbial growth as described in ASTM D2574-06.

0 = No bacterial recovery
1 = Trace contamination (1 to 9 colonies)
2 = Light contamination (10 to 99 colonies)
3 = Moderate contamination (> 100 distinct colonies)
4 = Heavy contamination (continuous smear of growth, colonies have grown together and are indistinguishable)
7.2. Dip Slides
Dip slides contain agar media on a paddle-like plastic support. Like agar plates, dip slides can be purchased with various types of media for growing different types of microorganisms. Some contain bacterial growth media on one side and fungal growth media on the other. Dip slides are packaged in a sterile container. When ready for use, the dip slide is removed from its container, dipped into the sample for several seconds, removed, and placed back into the sterile container. The dip slide is then incubated for a period of time (e.g. 48 - 72 h at 30°C). After the incubation period, the number of colonies on the dip slide is compared to a reference chart to determine the approximate concentration of bacteria and/or fungi in the sample.

7.3. 3M™ Petrifilm™ Count Plates
Petrifilm™ can often times be used as an alternative to agar plates. The analysis method for microbial growth is very similar to the procedure with agar plates. One advantage of Petrifilm is that they require less space. A 1 ml sample (or sample dilution) is added to the film, and the film is incubated. Various dyes incorporated into the film media allow for the visualization of colonies that develop during the incubation.
7.4. Microbial Activity Assays
The above mentioned tests rely on counting microbial colonies that develop on an agar plate, dip slide or Petrifilm™. Another way to determine the presence or absence of microbial growth is to monitor microbial activity. Basically, a product sample is put into a container with media which allows for growth of the microbes if they are present. This growth can be measured by monitoring a change in the media (e.g. CO₂ production, lower pH, turbidity, etc.). An example of this is BioLumix®, an automated system that can be used to monitor total microorganisms as well as specific types (e.g. Pseudomonas).

7.5. ATP (Adenosine triphosphate) Method
The methods listed above all require an incubation period (typically several days) before the results can be determined. There may be situations where a more rapid turnaround time is needed. One possible way to achieve this is to utilize an ATP based test. Including the sample preparation, results may be available within 5 minutes!

ATP is present in all living cells including bacteria and fungi. Even though a variety of factors affect ATP levels, the amount of ATP present in a sample can be used as an indicator of the amount of microbial growth present. The test method uses a chemical reaction between luciferin, luciferase and ATP. The reaction emits light which is measured by a photocell. The emitted light is directly proportional to the amount of ATP present.

The 3M™Clean-Trace™ ATP System is an example of an ATP testing device, and is shown below. A fixed volume of liquid is transferred to a tube containing all the reagents to run the test. After the reaction has occurred, the light emission is measured in the Clean-Trace luminometer.

Since the test method involves the detection of light, the transparency of the sample has a major impact on the efficacy of the test. Samples that are opaque are problematic. However, some companies claim to have ATP methods that will work with opaque products like paint. It is unclear whether or not these methods would work successfully for agrichemical formulations. To avoid false positives caused by accidentally picking up ATP through contact with the hands, breath etc. of the technician special care must be taken. Also it must be borne in mind that dead microbial cells can still release ATP.

3M, Petrifilm, and Clean-Trace are trademarks of the 3M Company
8. Microbiological Sources

8.1. Water
Not only is water the most abundant ingredient in SC, SE, SL and FS formulations, but it is also one of the most likely sources for microorganisms. The water used for the formulation of agrichemical products is typically derived from a local municipality (i.e. tap water), a well, or the manufacturing plant’s on-site water treatment facility. The water should be as clean as possible. Depending on the water quality, further processing may be required. For example, a water softener may be needed to reduce water hardness, or a deionization system may be required to remove ions. Regardless, all of these have the potential to support microbial growth, and they provide an ideal location for biofilm development.

Various methods can be used to reduce microbial growth in water systems including UV lights and 0.2 micron filtration. If the water quality parameters allow it, a disinfectant (e.g. sodium hypochlorite) may be added. It is highly recommended to check the microbiological water quality before and after water treatment. If biofilms develop in the water system, further treatment may be needed (Chapter 12).

8.2. Air
Microorganisms, particularly fungal spores, are present in air. Therefore, every effort should be made to minimize exposure of microbial susceptible products, raw materials and packaging material to air. For example, tank hatches should be kept closed unless there is a need to open them. When not in use, raw material drums should be kept closed and bags sealed.

8.3. Raw Materials
Many raw materials used in SC, SE, SL and FS formulations are susceptible to microbial growth. Principally all water based raw materials have the same potential risk as the finished formulations (e.g. antifoam dispersions, emulsifier or dispersing agent solutions, dyes for seed treatments, and thickening solutions). Even solid raw materials, powders and granules, may contain microorganisms in the form of bacterial endospores and fungal spores. Especially susceptible are those derived from natural sources or if their production uses biochemical processes (e.g. polysaccharide thickeners). Even raw materials of a similar chemical composition may have varying levels of microorganisms. Therefore, it is important to periodically check the raw materials, especially those that are known to be susceptible and capable of supporting high levels of microbial growth. It may be possible to work with the raw material supplier to improve the microbial quality of the raw material by adding a biocide, or by finding a like product that is certified to have a minimum level of bacteria and fungi.
8.4. Residual Product
A common, often overlooked source for microorganisms is residual product left in the manufacturing equipment within a production campaign. If microbial growth occurred in the final product and some of that product remains in the process, it will seed the next batch with microbes. Since the microbes are already adapted to growing in the product they can readily grow in the fresh product being made. Furthermore, the microbes may have some level of tolerance to the biocide being used to protect the formulation. This process can go on indefinitely unless a thorough cleaning is done to remove the entire residual product and/or an effective biocide treatment is used.

Similarly, packaging (e.g. an IBC) that once contained product with microbial growth can also serve as a source of microbes for fresh product added to the packaging. Therefore, use new containers. If IBCs or other containers have to be reused, make sure they have been thoroughly cleaned and disinfected to remove all the microbial growth. It has been demonstrated that IBCs and other containers made from plastic cannot be “microbially” cleaned and can therefore not be reused.

An area containing residual product and/or water that is sometimes overlooked are dedicated beads. To prevent residual product and water present in dedicated beads from becoming a source of microbes in the next product, two options are available for storage till the next manufacturing run:

- proper cleaning and complete drying of the beads and storage in a clean and dust free container till the next production run, or
- by storing beads under wet conditions after adding a biocide. Depending on the length of the storage period, a check of potential microbial growth before re-use is necessary because the biocide concentration drops with time.

Residues of a.i.’s and raw materials sticking to pallets, which are reintroduced in the manufacturing area can also serve as a source of microbial growth. Pallets need to be cleaned and thoroughly dried before re-use.
The production plants of crop protection formulations are typically not designed to operate under sterile conditions. Nevertheless, as some of the examples in the case histories show, plant equipment and design cannot be ignored.

Equipment design measures for improved cleaning efficiency are not only applicable to prevention of chemical cross contamination, but are equally important for preventing microbial growth. Both aspects must be considered if new production lines are being designed.

Formulation lines optimized to reduce microbial growth have:
• Clear process structure.
• As little piping and connections as possible.
• A piping system with gradient (≥ 4°) instead of horizontal construction to enable complete emptying/self-draining of the system.
• A piping system designed to allow the possibility of easy and fast dismantling with reasonable effort.
• Dedicated hoses for each product or at least product group with easy identification (e.g. labeling or bar codes).
• After use, all hoses must be drained and dried. Ideally hoses should be hung vertically or in the middle with the open ends pointing to the floor.
• No dead spaces and sacks especially for critical devices such as ball valves.
• Smooth, polished surfaces at least for critical equipment which allows faster, easier and better cleaning. Parts of filling modules, for example the buffer storage tank in front of the mass flow meter and its piping are considered critical equipment.
• Rubber hose usage reduced to an absolute minimum.
• Reduced usage of different diameter piping.
• Inspection of piping and other metallic equipment, which has been not been drained after use and has been out of commission for longer periods, has to be carried for corrosion caused by bacteria.

Filters are another area where microorganisms can accumulate and repeatedly inoculate product as it passes through. Therefore, filters (bags, cartridges) should be cleaned and/or replaced on a regular basis (e.g. between batches or campaigns).
One way to prevent or reduce microbial growth in the production process is to split feed the final product biocide to different parts of the system. This may be particularly helpful if certain parts of the process are highly susceptible to microbial growth. If this is done the biocide has to be the same biocide used for final product preservation. **Furthermore, the amounts of biocide added to the various components must not result in a final product having a biocide concentration exceeding the registration limit.**

*Remark:* A treatment of the formulation line by the described procedure is a preventive measure to avoid microbiological growth in the final product. It is not designed as a procedure to disinfect production lines.

The following process scheme shows a typical SC line. In this example, biocide is added to the slurry and thickener preparation vessels, and also to the finished product vessel. By adding biocide to the slurry and thickener preparation vessels the whole formulation line is treated with biocide.

The amount of biocide used in the process depends on the quality of the raw materials in terms of microbial growth, and on the conditions of the production line. A typical quantity of biocide used for the process treatment is half of the registered amount of biocide in the formulation. This means, if half of the amount is used for the process, the other half amount is added at the very end of the process just for treating the final product. The described procedure is usually more suitable if the level of microbial growth is low.
This diagram of the manufacture of a SC formulation shows where to add biocide during the process. The last addition of the biocide takes place during the final phase of the process.
It is well understood that crop protection production plants are not operated under sterile conditions and to ensure that products are free of microbial growth, sufficient plant hygiene is absolutely mandatory. It is important to avoid any continuous microbial infestation into the plant particularly in areas where raw materials are stored and where products are manufactured. Furthermore, environmental conditions suitable for bacterial and fungal growth must be avoided. Knowing where the microorganisms are present in the process can be very helpful in developing the means to control them.

11.1. Areas susceptible to microbial growth
Critical areas for microbiological growth are the same as the ones for chemical cross contamination:
- dead spaces
- seals
- porous or rough materials like rubber or plastics
- devices with large surfaces like filters, milling beads or gaps of colloid mills
- sharp angled pipes
- not properly cleaned surfaces, humid and residual production substances
- wet areas
- welded areas in pipes or vessels
- stored beads (used in SC, SE and FS milling processes)

Examples of some areas in formulation plants where improved plant hygiene is called for to greatly reduce or even eliminate microbial growth in water-based products

Dedicated formulation vessel was not cleaned after the production run; biofilm developed quickly. Chemical cross contamination is a non-issue in dedicated facilities, but this does not apply to the prevention of microbial growth.

Cleaning plastic piping and rubber sleeves is almost impossible. Recommendation: Avoid plastic piping.
Bacterial growth on the outside of the piping. Decontamination of the outside of piping must form a part of the routine cleaning procedure. Please note that there are two areas where water can be trapped and result in standing water. These must be drained to prevent creating an optimal environment for microbial growth.

Another area where microbial growth could readily develop: on piping near the ceiling and thus partially out of sight. This must not be overlooked when carrying out a decontamination.

Sight glass in transfer line needs to be dismantled and cleaned after the finish of each production run.

Poor welding of the coupling of a formulation line allowed for standing water in the pipe which is ideal for microbial growth.
Easily overlooked in a decontamination exercise: the inside of aspirators shown here with a build-up of microbial growth.

A filling lance for IBCs is stored after use in this wall mounted “bucket” without being drained and cleaned. Build-up of the residual liquid created ideal conditions for the growth of bacteria. Each time this lance was used, transfer of those bacteria to the next IBC took place.

The outside of this bulk container and feeding lines, used to transfer an aqueous raw material slurry to the end product, must be kept clean. The residue on the outside acts as a nutrient source for microorganisms. As is the case in this example, microbial growth could develop because of the high moisture level.

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Easily overlooked in a decontamination exercise: the inside of aspirators shown here with a build-up of microbial growth.

Bacterial growth on the base of a formulation vessel. With the appropriate plant hygiene, this can easily be avoided.
11.2. Testing for Microorganisms in the Production Facility.

Generally, the first step in testing for microorganisms in a production facility is to review the plant design via diagrams and a plant tour. A visual inspection of tanks and other accessible areas can be done at this time. If these areas appear dirty, inaccessible areas are probably worse.

When the plant is operating, samples may be taken at various stages of the manufacturing process. If the sample is taken from a sampling valve or tap, make sure that the tap is clean. Also, flush the tap for a period of time so that the sample is coming from the pipe or vessel, and not from some residual material that remained in the tap. One or more of the test methods described in section 7 can be used to test for the presence of microorganisms.

Depending on the severity of the problem, and the need to do a thorough examination, pipes may need to be disconnected, examined and sampled. A swab test can be used to check pipes or other surfaces especially for biofilms. A sterile cotton ball stick or something similar is wiped over the surface to be checked. The “contaminated” cotton ball stick is then wiped over an agar substrate. If no microbiology testing capability is available on-site, swabs can be purchased that will keep the sample intact until it can be tested (e.g. BBL™ CultureSwab™ Transport System). Alternatively, there are several fast tracking systems available used for hygiene control (e.g. ATP test, see 7.5).

Once the microbiology results are available, a map can be made which shows the sampling points and the observed levels of microbial growth at those points. This can often reveal where the “microbial hotspots” are in the facility and where critical control points are needed. Such cross checks may need to be carried out at a frequency depending on the severity of the problem.
To maintain an adequate level of plant hygiene, routine cleaning and disinfection will be needed. Different options are discussed below.

12.1. Mechanical Cleaning
Mechanical cleaning can involve high pressure spraying or scrubbing parts/surfaces with a brush. In some instances, it may be the only way to remove a biofilm or deposit. A high pressure cleaner is generally used for larger parts in the formulation plant such as vessels/tanks, agitators, large piping, and fixed connected charging systems. Scrubbing is usually implemented for smaller parts and surfaces. “Pigs” used for chemical cleaning may seem a useful tool for removing deposits, like biofilms. However, the danger is high that their use could result in distribution of the microbes further through the lines.

Usually the parts of the process that are most accessible are the parts that are most thoroughly cleaned. However, an effective cleaning may require the dismantling or opening of the most critical parts of the plant. Furthermore, it may be easier and more effective to replace some parts, such as filters, rather than attempt to clean them.

12.2 Chemical Cleaning
Not all parts and surfaces in a production facility are readily accessible. Therefore, chemical cleaners are often used to reach areas that are inaccessible to mechanical cleaning. Typical cleaning agents include:
- Caustic
- Acids
- Solvents
- Detergents

Some cleaning agents combine caustic or acids with detergents. The effectiveness of these cleaning agents can be enhanced by using them at a high temperature (≥ 70°C). In addition to cleaning, caustic or acids can also serve as disinfectants. A disinfectant is an agent, usually chemical, that is applied to surfaces to kill microorganisms.

Solvents are often used to dissolve chemicals that have poor water solubility. As such, they can be used to remove chemical deposits. Also, solvents are usually effective in killing microorganisms. Therefore, a chemical cleaning program that utilizes a solvent can also be an effective means for disinfection. However, like caustic or acids, solvents can have potential safety and handling issues that have to be taken into account (e.g. flammability, vapor pressure, inhalation concerns, chemical waste disposal etc.).
12.3 Chemical Disinfectants

Often mechanical and/or chemical cleaning is not sufficient to eliminate microorganisms from a process. Therefore, a disinfectant may be needed. As mentioned above, caustic, acid and solvents can serve as disinfectants. However, there are other chemical disinfectants available including the following:

- Sodium hypochlorite
- Hydrogen peroxide
- Peracetic acid
- Quaternary alkylated ammonium chloride mixtures (quats)

Each of these products has advantages and disadvantages. The resistance of biofilms and deposits requires that these products often have to be used at relatively high levels.

*Sodium hypochlorite* bleach is a well known disinfectant. It is frequently preferred because of efficacy, cost and handling safety. However, it can be very corrosive to metal surfaces and reactive with a variety of compounds. If the hypochlorite or hypochloric acid is consumed by these compounds, there may not be enough left to kill the microorganisms. Sodium hypochlorite is sold at different concentrations so the amount used will depend on the concentration of the product. Generally, an active concentration of 100 – 150 ppm as Cl₂ is recommended for disinfection.

*Hydrogen peroxide* is another common disinfectant especially for DI water systems. Typical use concentrations are > 0.5 % active hydrogen peroxide. One advantage of using hydrogen peroxide is that it degrades to water and oxygen, and therefore should present no issues with regard to waste disposal. However, depending on the concentration purchased, safety and handling may be more problematic than with bleach. Also many bacteria produce an enzyme called catalase, which degrades hydrogen peroxide. This is one reason low levels of peroxide may be ineffective.

*Peracetic acid (PAA)* (a combination of acetic acid and hydrogen peroxide) is also commonly used as an industrial disinfectant. As sold, PAA products usually contain hydrogen peroxide as well. This combination offers some of the same advantages and disadvantages as peroxide (i.e. good environmental profile, potential handling and safety issues). As with the other disinfectants, this product is purchased at a higher concentration (e.g. 5% PAA, 20% hydrogen peroxide), and diluted (e.g. 1:100) before use.

*Quaternary ammonium compounds (quats)* are often used as surface disinfectants. They are effective against bacteria and fungi at concentrations > 0.5 %. However, the use of quats can cause significant foaming. They are commonly used to wipe down and disinfect surfaces. They are used less often on an industrial scale to disinfect piping, etc. One reason is that the disposal of quats can be difficult as they tend to be detrimental to waste treatment systems.
12.4. Hot water or steam

Hot water or steam can be a very effective disinfectant. **For hot water, typically temperatures of at least 70°C with a minimum of 1 hour contact time are needed.** The higher the temperature of the water, the more effective it will be, and the contact time may be reduced. One advantage: hot water/steam is that there is no added chemical (or post rinses) that has to be disposed of in a waste treatment system. However, there is an energy cost, and potential safety issues especially with the use of steam. For treatment with superheated steam, the line must be pressure proof. **Please be aware that most formulation lines are not pressure proof.**

Most importantly for the disinfection by high temperature is that all parts of the line are heated up to the necessary temperature. Based on the heat transfer through surfaces, the real temperature at a pipe surface or at surfaces of a filter could initially be much lower than the temperature of the heat transfer media (steam or water). Therefore, it must be ensured that the time of treatment at the high temperature is long enough to allow all parts to reach the correct temperature for disinfection. In addition the time for heating the system up to the correct temperature must be taken into account, and added to the actual disinfection time.

12.5. Treatment Strategy

The type of cleaning and disinfection that a plant utilizes, and its frequency, will depend on the severity of the microbial problem. Every plant is different, and what works for one, may not work for the other. Furthermore, other factors (e.g. waste disposal limitations) may affect the decision process on what to use, and how to use it.

Relying solely on one cleaning agent or one disinfectant is typically not as effective as using a combination. This combination may involve a sequence of two cleaning agents with disinfectant activity (caustic and acid), or a cleaning agent followed by a disinfectant. For example, combinations of sodium hydroxide followed by sodium hypochlorite can be very effective.

An effective cleaning may require the following:

- Mechanical cleaning to remove visible deposits and residual product. (e.g. high pressure washing of vessels). This should be drained before the next step.
- Chemical cleaning especially for areas that cannot be readily accessed (e.g. pipes, pumps, etc.). This may involve caustic, acids, solvents or detergents. After cleaning, drain the residual cleaning agent.
- Rinse the areas affected by the cleaning agent with clean water.
- If needed, add a chemical disinfectant (e.g. sodium hypochlorite) and circulate to critical areas. Drain the disinfectant.
- Rinse with clean water. Several rinses are likely to be needed to remove residual disinfectant, and prevent product contamination.

**Hot water or steam may be substituted in place of the chemical cleaning/disinfectant agents.**
12.6. Drying
Microorganisms need water to grow. Therefore, drying can be an effective method to inhibit microbial growth. This can be particularly important if the plant is not running for more than 3 or 4 days, and there are areas in the process where water can accumulate. If possible, areas of water accumulation should be drained. If there are areas that cannot be drained, some residual disinfectant or biocide may have to be added to prevent microbial growth. However, this preserved water will need to be flushed out with clean water before the next production batch.

13. Rework of formulations containing microorganisms

Whether or not a product containing microbial growth can be reworked depends on various factors. If the product has been irreparably damaged then it needs to be disposed of in a safe manner. A product batch with microbial growth should not be remediated by blending with another batch free of microorganisms. Most likely this will result in more batches containing more microbes.

Rework of product with microbial growth at the plant of a toll manufacturer can only be carried out after approval by the client and with an agreed rework procedure (most clients will provide this procedure in written form).
ASTM: American Society for Testing and Materials

Bacteria: small (typically 1 to 5 micron in size), single-cell organisms that reproduce by dividing in two

Biocide: a chemical used to kill microorganisms and/or inhibit microbial growth

Biocide Tolerance: a condition in which microorganisms are no longer killed or inhibited by a biocide

Biofilm: a slimy layer on a surface composed of microorganisms, nutrients, metabolites and water

CFU: Colony Forming Unit – individual or clumps of cells / spores that grow into a colony on a solid media surface (e.g. agar plates, Petrifilm™ or dip slides)

Dip Slides: a paddle like structure containing media for growing microorganisms, commonly used to test for the presence of microbial growth in chemical products

Disinfectant: an agent, usually chemical, that is applied to surfaces to kill microorganisms

ECPA: European Crop Protection Association

Enzymes: proteins produced by bacteria and fungi to perform a biochemical function (e.g. to degrade Xanthan Gum)

FS: Flowable formulations used for seed treatments.

Fungi: microorganisms that consist of two groups - yeasts (small single cells that reproduce by budding) and molds (grow in threadlike filaments and form spores)

Hygiene: the practice of keeping a manufacturing plant or process clean

Micron: unit of length - one-thousands of a millimeter (0.001 mm) or approximately 1/25,000 inch. This unit is abbreviated as µm, and nowadays often referred to as micrometer

Microorganisms (microbes): small organisms (single organisms are usually visible only with a microscope)

MSCSG: Manufacturing and Supply Chain Steering Group, group in ECPA involved with global manufacturing and supply chain policies affecting our industry
Seed Treatment Formulations (FS = flowable concentrate for seed treatment): stable water-based flowable concentrates for seed treatments. FS formulations are often applied in undiluted form. The formulation is very similar to that of SCs, but contains a sticker to help the FS to adhere better to the seeds.

Spores: small bodies produced by molds and bacteria (endospores) that allow the microorganisms to survive harsh conditions (e.g. drying).

Soluble Liquid (SL): an agrichemical formulation in which the active ingredient as its salt is completely dissolved.

Suspension Concentrate (SC): an agrichemical formulation in which the active ingredient(s) as solid particles are dispersed in a continuous aqueous phase.

Suspo-Emulsion (SE): an agrichemical formulation in which a lipid soluble active ingredient is present emulsified in an organic phase besides the aqueous phase with solid particles.
Prevention and Control of Microbial Growth in Water-Based Crop Protection Formulations: Self-Assessment Checklist

This self-assessment checklist will help manufacturers and their toll manufacturers to determine whether their manufacturing processes and technical equipment comply with principles of prevention and control of microbial growth in water-based crop protection formulations. A negative reply to any of the questions in the checklist should have a corresponding action plan for improvement or an explanation why an improvement is not necessary.

This checklist can also be used as the Microbial Growth Prevention and Control section of a client’s toller / contractor Contamination Prevention Audit checklist.

The frequency of Microbial Growth Prevention and Control self-assessments and/or audits of the facilities has to be determined on an individual basis by each client and their toller/contractor based on their own risk assessments. The frequency must be adapted when changes occur that could potentially result in increased microbial growth.

Self-assessments and audits will be required:
- In case the product mix has been changed and a new water-based formulation has been added to the portfolio.
- After completion of the action plan which resulted from the initial self-assessment/audit to ensure that the non-conformities have been corrected.
## 1. Management Responsibility

### 1.1 Knowledge of risks associated with microbial growth in water-based agrichemical formulations
- Is knowledge / experience available at your site regarding prevention of microbial growth in water-based (aqueous) formulations?

*Please provide details:*

### 1.2 Standards
- Does your site have a company standard / guideline / Standard Operating Procedures / company policy covering “Prevention of Microbial Growth in Water-Based Crop Protection Formulations”?

### 1.3 Responsible person
- Do you have an appointed person in your organization for the implementation of “Prevention of Microbial Growth in Water-Based Crop Protection Formulations” methodology?
- Name (s)?
- In this role since?
- Is this person also responsible for implementation of prevention of chemical cross contamination?
<table>
<thead>
<tr>
<th>Management Responsibility (continued)</th>
<th>Yes</th>
<th>No</th>
<th>Comments/Details/Proposed Action Plans</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.4. Training and awareness</strong></td>
<td></td>
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<tr>
<td>Does “Prevention of Microbial Growth in Water-Based Crop Protection Formulations” form an integral part of your Quality Awareness training?</td>
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<tr>
<td>• How often is this training module given?</td>
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<tr>
<td>• Do you provide this training to both existing and new personnel (including temporary seasonal personnel)?</td>
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</table>
2. **Product Mix**

<table>
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<tr>
<th></th>
<th>Yes</th>
<th>No</th>
<th>Comments/Details/Proposed Action Plans</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2.1.</strong> Which water-based formulations do you manufacture at your site?</td>
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<tr>
<td>• Soluble liquids (SLs)?</td>
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<tr>
<td><em>Please describe</em> and specify a.i. contents:</td>
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<tr>
<td>• Suspension Concentrates (SCs)?</td>
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<tr>
<td><em>Please describe:</em></td>
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<tr>
<td>• Suspoemulsions (SEs)?</td>
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<td><em>Please describe:</em></td>
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<td>• Seed treatments formulations (FS)?</td>
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<td><em>Please describe:</em></td>
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<tr>
<td><em>This information will also be required to determine cleaning levels needed to prevent chemical cross-contamination.</em></td>
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</table>

<p>| <strong>2.2.</strong> Water Source |     |    |                                       |
| • Please describe the water source for use in these formulations, i.e. used as process water. |     |    |                                       |
| Is the water treated chemically or purified (deionization, reverse osmosis, filtration, UV lights, etc.) before use in the production of agrichemicals? |     |    |                                       |
| <em>Please describe how:</em> |     |    |                                       |</p>
<table>
<thead>
<tr>
<th>Product Mix (continued)</th>
<th>Yes</th>
<th>No</th>
<th>Comments/Details/Proposed Action Plans</th>
</tr>
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<tbody>
<tr>
<td><strong>2.3. Biocides</strong></td>
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<tr>
<td>• Which biocides are used in each of the formulations mentioned in 2.1?</td>
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<tr>
<td><em>Please provide a detailed list:</em></td>
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<tr>
<td>• What is the concentration of these biocides in the formulations listed above?</td>
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<td><em>Please specify for each formulation:</em></td>
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<td></td>
<td><strong>3. Microbiological Screening</strong></td>
<td>Yes</td>
<td>No</td>
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<td>---------------------------------</td>
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</table>
| 3.1 | Do you have a **cleaning program in place** for chemical cross contamination, microbial growth, or other reasons?  
*Please provide details on methods/chemicals used:* |     |    |                                      |
|     | • Do you only clean between campaigns?  
• Or, do you clean also between batches?  
☒ Between each batch or between …… batches? |     |    |                                      |
| 3.2 | Do you have a map of the production facility in which the areas susceptible to microbial growth (“hot” spots) are clearly marked? |     |    |                                      |
| 3.3 | Do you have visual inspections checking for microbial growth at these “hot” spots?  
• Before the start of a campaign of a water-based formulation?  
• Also during the actual manufacturing process? |     |    |                                      |
### Microbiological Screening (continued)

| 3.4. | Do you have a routine microbiological screening program in place on your site?  
• Are the production and packaging lines checked for microbial growth?  
• Is the **water supply** checked for the presence of microbes?  
• Are raw **materials and intermediates** checked for the presence of microbes?  
• Do you check **packaging material** for the presence of microbes?  
• Do you check **materials that need to be reworked** for the presence of microbes?  

*Please give details:*  

• Do you check **materials that will be recycled** for the presence of microbes?  

*Please give details:*  

☐ Before storage?  
☐ Before re-introduction in the process?  
• Do you check finished product for the presence of microbes?  

*Please give details:* | Yes | No | Comments/Details/Proposed Action Plans |