

## Appendix C

### Checklist (according to the "List of approved in-can preservatives") pursuant to the Basic Award Criteria and the currently approved preservatives for the evaluation of substances as part of the admission process for other in-can preservatives.

Chemical name of the substance: \_\_\_\_\_

CAS no.: \_\_\_\_\_

Synonyms: \_\_\_\_\_

Trade name: \_\_\_\_\_

Only those substances (active substances or biocides) may be used for which an active substance dossier for preservatives for products during storage (product-type 6) according to the Biocidal Product Regulation (BPR, Regulation (EU) 528/2012) has been submitted. If the substance is included in the Union list of approved substances for product-type 6 after a corresponding evaluation, only the data according to Annex 1 to the checklist and Annexes 4a-4c to the checklist must be submitted to the Blue Angel for further evaluation as part of the admission process for other in-can preservatives for paints/varnishes, floor-covering adhesives and sealants. The same also applies to substances and substance combinations on the "List of approved in-can preservatives" that have already been evaluated by the German Environment Agency.

#### 1. Data on the toxicology of the substance<sup>1,2</sup>

a) Information on the following points is enclosed as an Annex:

- ☐ Inhalation toxicology;
- ☐ Carcinogenicity;
- ☐ Sensitizing effect;
- ☐ Contribution to systemic toxicity after percutaneous absorption;
- ☐ Risks during pregnancy;
- ☐ Germ cell mutagenicity;
- ☐ Reasons for the establishment of a MAK value;
- ☐ Other.

<sup>1</sup> By submitting as much data as possible in accordance with Annex 1 to the checklist, including the associated test report, it will avoid the need for time-consuming follow-up questions and clarifications. The submission of a summary of the data in the form of an evaluation dossier that was produced by a reputable, independent testing institution will also simplify the process considerably.

<sup>2</sup> Not required for substances already included in the "List of approved in-can preservatives" (a-t) or substances included in the Union list of approved substances for product-type 6.

- b) Has this substance been classified by the EU and have corresponding risk reduction measures been derived?
- According to Regulation (EC) No. 1907/2006 (existing substances): ☐ Yes / ☐ No  
If Yes, with what results? (Please add a brief description)
  - According to Regulation (EC) No. 1907/2006 (new substances): ☐ Yes / ☐ No  
If Yes, with what results? (Please add a brief description)
  - Other national or international classifications: ☐ Yes / ☐ No  
If Yes, with what results? (Please add the source and a brief explanation)
- c) Has the substance been classified in accordance with the regulations on hazardous substances (CLP Regulation (EC) No. 1272/2008, Regulation (EC) No. 1906/2006 concerning the Registration, Evaluation, Authorization, and Restriction of Chemicals, in short REACH; TRGS 905 and others) (legal classification)? ☐ Yes / ☐ No
- If Yes, please add corresponding information in a concise form
  - If No, which self-classification was made?  
(Please add corresponding information in a concise form)
- d) Has the substance been evaluated and classified under the German Biocide Act (in accordance with the Biocidal Products Regulation (EU) No. 528/2012)? ☐ Yes / ☐ No
- If Yes, with what results? (Please add a brief description)
- e) In which water hazard class (WHC) according to AwSV (German Ordinance on Facilities Handling Substances that are Hazardous to Water) is the substance classified?  
(Please add a justification for the WHC classification)
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- f) Could the substance have a negative impact on health and the environment as a result of interactions with other ingredients or auxiliary substances in the product due to its structural characteristics? ☐ Yes / ☐ No
- If Yes, please add corresponding information in a concise form

## 2. Exposure measurements for the substance

Exposure data is required for the evaluation for inclusion on the "List of approved in-can preservatives". For this purpose, the preservative must be added to an emulsion paint without preservatives (the starting formulation) and the emissions are then measured in a test chamber or test cell. The test conditions are enclosed in Annex 1 to the checklist.

The application concentration of the preservative that guarantees its effectiveness as a preservative should be selected (this application concentration is determined in the test for

assessing the product's fitness for use according to Paragraph 4, sentence 4 and Annex 4). As a concentration range is usually derived for the required preservative function, it is recommended that three measurements with different concentrations are completed. Depending on the available data, the results should be given as an area-specific emission rate in [ $\mu\text{g}/\text{m}^2 \text{ h}$ ] or as a concentration in [ $\mu\text{g}/\text{m}^3$ ]. The evaluation must be carried out in accordance with Annex 2.

### **3. Evaluation of exposure and derivation of a comparative value to the "Indoor Air Guide Value (RW I)"**

Has the tested substance produced a value above the maximum workplace concentration (MAK value)? ☐ Yes / ☐ No

a) If Yes, Please add corresponding information in a concise form:

Evaluation: Derive a comparative value to the Indoor Air Guide Value "RW I" in accordance with [1] as a value for an acceptable indoor air concentration for the substance according to Annex 3

b) If No:

Has the tested substance produced a value above the maximum daily intake value, e.g. ADI, DTA or TDI value? ☐ Yes / ☐ No

- If Yes, please add corresponding information in a concise form:

Please carry out an evaluation in accordance with Annex 2.

- If No, It is recommended that a comparative value to the Indoor Air Guide Value "RW I" is derived in accordance with [1] as a value for an acceptable indoor air concentration for the substance according to Annex 3 based on NO(A)EL or LO(A)EL values. This value should be compared with the concentration value in [ $\mu\text{g}/\text{m}^3$ ] from the emission measurements carried out in accordance with the conditions defined in Annex 1. The value measured in accordance with the conditions defined in Annex 1 must be lower than the comparative value derived for the Indoor Air Guide Value "RW I".

If no NO(A)EL or LO(A)EL values are available for the substance being tested, they should be determined experimentally by the applicant using suitable methods.

### **4. Determining the effective limit and maximum permissible content in the wall paint**

To determine the minimum quantity of the preservative preparation, the applicant shall conduct a test of the product's fitness for use with at least 3 different concentrations of the

preservative in accordance with the rules defined in the enclosed Annexes 4a-4c3. The applicant shall submit the results to the German Environment Agency (UBA) Specialist Department III 1.4 for evaluation by BAM (Federal Institute for Materials Research and Testing) and the UBA.

## **5. Literature**

[1]: Seifert, B.: Richtwerte für die Innenraumluft: Basisschema. Bundesgesundheitsblatt 11/96 (Guide Values for Indoor Air: Basic Scheme. Federal Health Bulletin 11/96).

[2]: DIN EN ISO 16000-9.

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<sup>3</sup> From an independent testing laboratory or BAM

## **Annex 1 to the checklist:**

### **Completion of the emission measurements**

The emission measurements to determine the content of the preservative in indoor air are based on DIN EN 16516.

In particular, this means that the test chambers/cells must comply with the following requirements:

- High-purity air supply (free of VOC and dust);
- High-purity water supply;
- Chamber/cell walls made of glass or stainless steel;
- No use of sealing materials where possible
- Control of jacket temperature is recommended.

And the following test conditions must also be complied with:

- Temperature (T)  $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ;
- Relative humidity (RH)  $50 \% \text{ r.F.} \pm 5 \% \text{ r.F.}$
- Air flow rate  $0,1 - 0,3 \text{ m/s}$
- Air exchange rate [1/h]:  $0,5$ ;
- Loading factor [ $\text{m}^2/\text{m}^3$ ]:  $1,4$ ;
- Carrier material: Glass slide;
- Single coat;
- Coat quantity by area [ $\text{g}/\text{m}^2$ ]:  $150 \pm 25$ ;
- Paint application device: Rakel

The test should be carried out using the sample recipe with the loading for walls ( $1.0 \text{ m}^2/\text{m}^3$ ) and ceilings ( $0.4 \text{ m}^2/\text{m}^3$ ) and an air exchange rate of 0.5. The production date must not be more than 8 weeks ago.

Measurements must be taken on days 1, 3, 7, 14 and 28 as double determinations.

The test chamber or test cell measurements must be carried out continuously throughout the entire test period; it is not permitted to remove the samples.

**Annex 2 to the checklist:**  
**Exposure Evaluation**

**Evaluation of the data in case a single value exceeds the maximum daily intake value (taking, for instance, ADI, DTA or TDI as a basis)**

**- Comparison of Data by the use of Emission Factors -**

a) Determination of the emission factor E in [ $\mu\text{g}/\text{m}^2 \text{ h}$ ]:

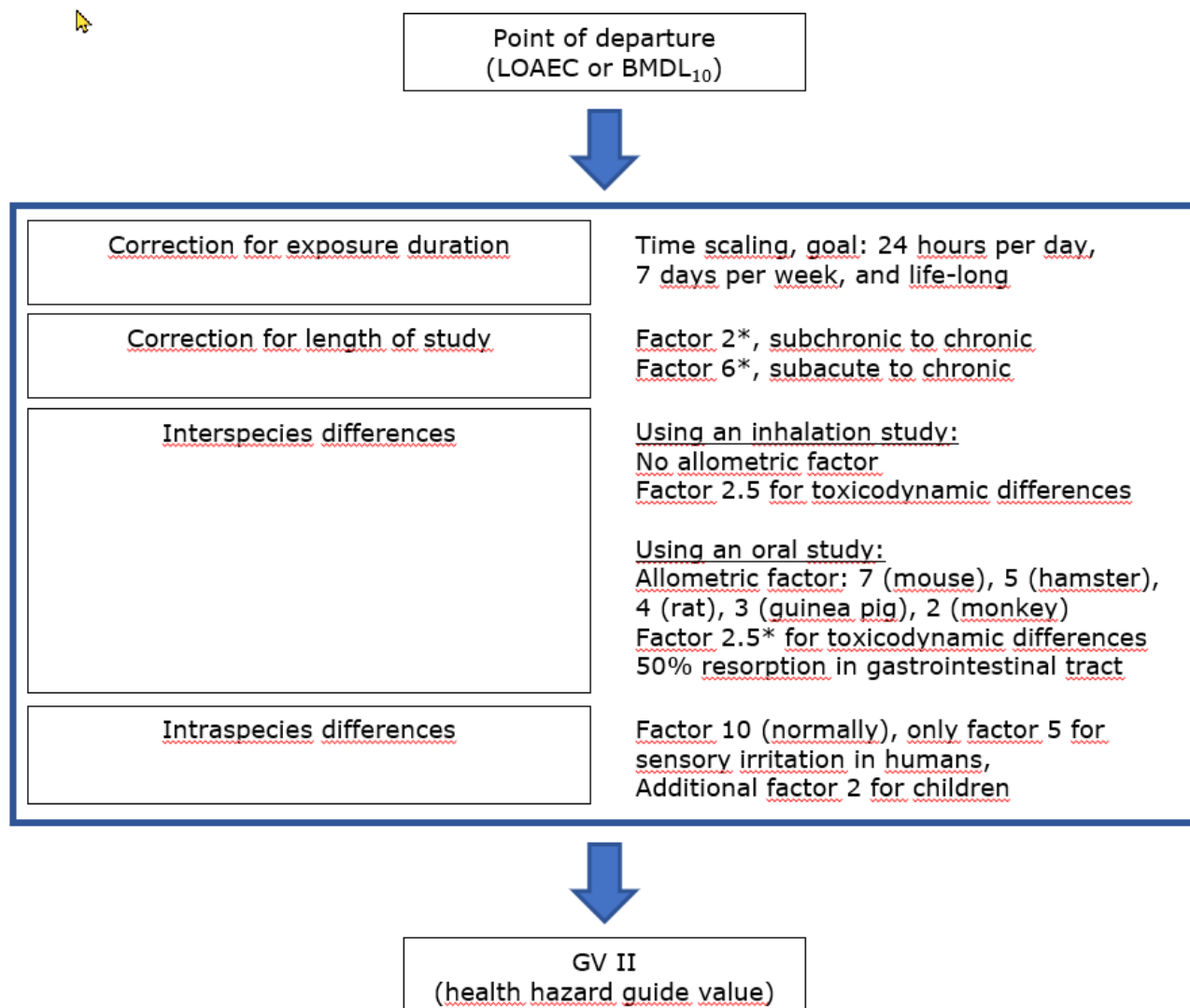
In the unfavourable case (breathing rate of an infant  $A = 0.94 \text{ m}^3 \text{ d}^{-1} \text{ kg}^{-1}$ , loading  $F/V = 2$  and air change rate  $n = 0.2 \text{ h}^{-1}$  – corresponds to the emission surfaces walls, ceilings and floor in a small room) and under the condition that the inhalation path may not contribute more than 10% to the total intake the following applies to maximum allowable emission rate:

$$E [\mu\text{g}/\text{m}^2 \text{ h}] = \text{ADI} [\mu\text{g}/\text{kg d}] \times 0.01.$$

b) Comparison of the measured value in [ $\mu\text{g}/\text{m}^2 \text{ h}$ ] with the value derived under a).

**Annex 3 to the checklist:****Derivation of a Reference Value by analogy with the Guidance Value „RW I“**

Reference Value to the Guidance Value II (RW II)



The reference value compared to guidance value „RW I“ amounts to 10 % of guidance value „RW II“.

## Appendix D

### Method for determining the effective limit and maximum permissible content in paint/varnish (biotest)

To determine the minimum quantity of the preservative preparation, the applicant shall conduct a test of the product's fitness for use with at least 3 different concentrations of the preservative in accordance with the rules defined in the enclosed Annexes 4a-4c<sup>4</sup>. The applicant shall submit the results to the German Environment Agency (UBA) Specialist Department III 1.4 for evaluation by BAM (Federal Institute for Materials Research and Testing) and the UBA.

#### Annex 4a to the checklist:

#### Carrying out a test of the product's fitness for use using bacteria

The applicant shall carry out a test of the product's fitness for use using the following method to determine the minimum quantity of the biocidal product <sup>5,6</sup> required:

#### A laboratory method for determining the concentration of a biocidal product that is required to combat bacteria in low emission and low pollutant paints and varnishes according to DE-UZ 12a

##### 1. Scope

The method can be used to test how effective biocidal products are at preventing the growth and survival of damaging organisms in aqueous, polymer-based paints and varnishes. This method is also used for the inclusion of biocidal products in the list of approved in-can preservatives and for verifying the suitability of other mixtures.

##### 2. Health note

National health regulations must be observed during the execution of the test. This also applies to EC Directive 200/54/EC "protection of workers from risks related to exposure to biological agents at work". When handling paints/varnishes and biocides, the instructions in the corresponding safety data sheets and product information brochures for the safe use of the product must be observed.

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<sup>4</sup> Test to be completed by a testing institution accredited according to DIN EN ISO/IEC 17025

<sup>5</sup> Product-type 6 according to the Biocidal Products Regulation (EU No. 528/2012)

<sup>6</sup> Only those substances or substance combinations stated in the list of approved in-can preservatives are permitted.



### 3. Instruments and nutrient media

- Suitable sterile, screw cap containers (100 ml);
- Sterile measuring pipettes, nominal volume 1.0 ml and 5.0 ml;
- Sterile glass or plastic petri dishes, diameter 90 or 100 mm;
- Sterile diluent; e.g. distilled water (for agar) according to ISO 3696;
- Physiological saline (to rinse and dilute bacterial cultures);
- Scales;
- Pipette and 0.1 cm<sup>3</sup> sterile tips;
- Bunsen burner;
- Incubator, thermostatically controlled (30°C +/2°C);
- Autoclave;
- Sterile inoculating loops or needles;
- Sterile spatulas;
- Sterile nutrient media for the corresponding micro-organisms, composition and production (see Annex 1);
- pH meter;
- Bacterial stock cultures;
- Test tubes;
- Test tube racks;
- Test tube shaker;
- Haemocytometer (depth 0.02 mm);
- Microscope.

### 4. Test organisms

The following bacteria should be used for the bacterial load test:

#### **Bacteria:**

<i>Alcaligenes faecalis</i>	BAM 604 or DSM 6174 or ATCC 35655
<i>Escherichia coli</i>	BAM 605 or DSM 787 or ATCC 11229
<i>Pseudomonas aeruginosa</i>	BAM 60 or DSM 939 or ATCC 15442
<i>Pseudomonas putida</i>	BAM 644 or DSM 291T or ATCC 12633
<i>Pseudomonas stutzeri</i>	BAM 607 or DSM 5190T or ATCC17588

Other bacteria that continuously lead to infections can be additionally used in a separate inoculation suspension. It is necessary to carry out a molecular and biological characterisation of these organisms (which must be available as pure cultures) to clearly determine the bacterial strain being used. The organisms should be stored at a

recognised culture collection.

## 5. Method

Inspect the materials received for testing before starting the test. To do this, use a sterile inoculating loop to spread the paint/varnish onto nutrient agar plates and check for any possible growth.

It is necessary to check both unaged samples and also artificially aged samples (see 5.2). The Pass/Fail criteria must be met in both cases (see 5.5).

### 5.1. Preparation of the inoculation suspension

5.1.1. Spread the test organisms taken from a continuous culture (e.g. cryo culture) onto nutrient agar plates and incubate them for 18 - 24 hours at  $30\text{ °C} \pm 2\text{ °C}$ <sup>7</sup>.

5.1.2. Prepare separate suspensions for each bacterial strain by wetting the growing surface on the nutrient agar plates using a sterile diluent, e.g. physiological saline, and carefully wash off the bacterial growth using a sterile cotton swab.

5.1.3. The number of organisms in each suspension is determined using a suitable haemocytometer (e.g. Thoma chamber, chamber depth 0.02 mm). The cell count of the individual bacteria suspensions should be  **$1 \cdot 10^8 - 5 \cdot 10^8$  KBE/ml**.  
The prepared inoculation suspension must be used on the same day and should be kept in a refrigerator until used.

5.1.4. In order to prepare a mixed suspension, combine and mix together identical volumes of each bacterial suspension. The cell count should also be  **$1 \cdot 10^8 - 5 \cdot 10^8$  KBE/ml**.

5.1.5. Check the defined cell count for the mixed suspension by spreading an appropriately diluted mixed culture onto CASO agar and determining the number of colony-forming units.

### 5.2. Artificial ageing

5.2.1. Add the biocidal product to the paint/varnish at appropriate concentrations for the test series, vigorously mix the samples and store at room temperature for at least

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<sup>7</sup> If the laboratory also uses its own test strains separately, the incubation time can be amended for these bacteria if necessary. The amended parameters used for testing the laboratory's own strains must be stated in the test report.

two days.

- 5.2.2. Weigh out suitable portions (e.g. 50 g or 100 g) of the paint/varnish into sterile screw cap containers and close them tightly.
- 5.2.3. Six non-preserved samples should be used as control samples. For this purpose, inoculate three samples with bacteria (positive control) and leave the remaining three samples uninoculated (negative control or retained sample).
- 5.2.4. Store the samples for 4 weeks at  $40\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$  or alternatively 18 weeks at  $30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .
- 5.2.5. After the 4-week ageing process, carry out the bacterial load test as described in Paragraph 5.3.

### **5.3. Bacterial load test**

- 5.3.1. Inoculate each sample (except for the negative control) with the same volume of mixed suspension equivalent to 1.0 % of the sample weight. Mix the sample thoroughly using a sterile spatula and then screw the cap onto the container in such a way that oxygen can still enter the container.
- 5.3.2. In order to determine the initial bacterial load for all samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Then incubate the plates at  $30\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for a maximum of 3 days<sup>7</sup>. Use a suitable method to determine the number of colony-forming units.
- 5.3.3. In order to check the vitality of the bacteria being used, spread and count the mixed suspension as described under Paragraph 5.3.2.
- 5.3.4. Incubate all samples for 7 days at  $30\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ .
- 5.3.5. In order to determine the cell count in the samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Determine the colony-forming units after incubating the plates at  $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for a maximum of 3 days<sup>7</sup>.
- 5.3.6. Repeat steps 5.3.1 to 5.3.5 at weekly intervals until at least 3 cycles have been

completed. The test is concluded after a total incubation time of 6 weeks at 30 °C  $\pm$  2 °C. Determine the final number of colony-forming units.

- 5.3.7. In order to determine the effectiveness of different biocidal concentrations depending on the incubation times, additional cell counts can be determined, e.g. 1 and 3 days after inoculation.

#### **5.4. Validity of the test**

The test is valid if the growth capacity of the inoculate can be verified on CASO agar in petri dishes (see 5.1.6).

#### **5.5. Pass/Fail criteria**

- 5.5.1. The curative effect of the biocide in the paint/varnish at the added concentration is verified if the cell count is  $\leq$  1000 CFU/ml at the end of the test.

- 5.5.2. The effectiveness of a tested biocide cannot be verified if the cell count in the biocide-free varnish after inoculation is  $<$  1000 CFU/ml at the end of the test.

#### **5.6. Verification of a significant difference**

Use suitable statistical measures to verify any significant differences between the cell count in the samples treated with in-can preservatives and in the untreated samples.

## **Annex 1 Nutrient medium**

### **Nutrient agar CASO**

Typical composition (g/L):

- Peptone from casein 15,0
- Peptone from soybean 5,0
- Sodium chloride 5,0
- Agar 15,0
- pH 7,3 ± 0,2

### **Preparation**

Suspend 40 g of nutrient agar in 1 L of distilled water, stir until completely dissolved and then autoclave for 15 minutes at 121°C.

**Annex 4b to the checklist:****Carrying out a test of the product's fitness for use using yeasts**

The applicant shall carry out a test of the product's fitness for use using the following method to determine the minimum quantity of the biocidal product<sup>5</sup> required:

**A laboratory method for determining the concentration of a biocidal product that is required to combat yeasts in low emission and pollutant paints and varnishes according to DE-UZ 12a**

**1. Scope**

The method can be used to test how effective biocidal products are at preventing the growth and survival of damaging organisms in aqueous, polymer-based paints and varnishes. This method is also used for the inclusion of biocidal products in the list of approved in-can preservatives and for verifying the suitability of other mixtures.

**2. Health note**

Before starting any test, ensure that national public health regulations and EC Directive 200/54/EC "protection of workers from risks related to exposure to biological agents at work" are being observed. When handling paints/varnishes and biocides, the recommendations in the corresponding safety data sheets and product information brochures for the safe use of the product must be observed.

**3. Instruments and nutrient media**

- Suitable sterile screw cap bottles (100ml);
- Sterile measuring pipettes, nominal volume 1.0 ml and 5.0 ml;
- Sterile glass or plastic petri dishes, diameter 90 or 100 mm;
- Sterile diluent; e.g. distilled water (for agar) according to ISO 3696;
- Physiological saline (to rinse and dilute the yeast cultures);
- Scales;
- Pipette and 0.1 ml sterile tips;
- Incubator, thermostatically controlled ( $30 \pm 2^{\circ}\text{C}$ );
- Autoclave;
- Sterile inoculating loops or needles;
- Sterile spatulas;
- Sterile nutrient media for the corresponding micro-organisms, composition and production (see Annex 1);
- Yeast stock cultures;

- Test tubes;
- Test tube racks;
- Test tube shaker;
- Haemocytometer (depth 0.1 mm);
- Microscope.

#### 4. Test organisms

The following yeasts should be used for the yeast load test:

##### Yeasts:

<i>Candida boidinii</i>	BAM 649
<i>Yarrowia lipolytica</i>	BAM 641
<i>Candida valida</i>	BAM 643

Other yeasts that continuously lead to infections can be additionally used in a separate inoculation suspension. It is necessary to carry out a molecular and biological characterisation of these organisms (which must be available as pure cultures) to clearly determine the yeast strain being used. The organisms should be stored at a recognised culture collection.

#### 5. Method

Inspect the materials received for testing before starting the test. To do this, use a sterile inoculating loop to spread the paint/varnish onto nutrient agar plates and check for any possible growth.

It is necessary to check both unaged samples and also artificially aged samples (see 5.2). The Pass/Fail criteria must be met in both cases (see 5.5).

##### 5.1. Preparation of the inoculation suspension

5.1.1. Spread the test organisms taken from a continuous culture (e.g. cryo culture) onto nutrient agar plates and incubate them for 24 - 48 hours at  $30\text{ °C} \pm 2\text{ °C}$ .

5.1.2. Use the yeasts from these plates to inoculate fresh nutrient agar plates (2nd subculture) and incubate them for 24 - 48 hours at  $30\text{ °C} \pm 2\text{ °C}$ . Only these cultures are used for the yeast load test.

5.1.3. Prepare separate suspensions for each yeast strain by wetting the growing surface on the nutrient agar plates using a sterile diluent, e.g. physiological saline, and carefully wash off the yeast growth using a sterile cotton swab.

5.1.4. The number of organisms in each suspension is determined using a suitable haemocytometer (e.g. Thoma chamber, chamber depth 0.1 mm). The cell count of the individual yeast suspensions should be  **$8 \cdot 10^7 - 2 \cdot 10^8$  - KBE/ml**.

The prepared inoculation suspension must be used on the same day and should be kept in a refrigerator until used.

5.1.5. In order to prepare a mixed suspension, combine and mix together identical volumes of each yeast suspension. The cell count should also be  **$8 \cdot 10^7 - 2 \cdot 10^8$  - KBE/ml**.

5.1.6. Check the defined cell count for the mixed suspension by spreading and checking an appropriately diluted mixed culture.

## **5.2. Artificial ageing**

5.2.1. Add the biocidal product to the paint/varnish at appropriate concentrations for the test series, vigorously mix the samples and store at room temperature for at least two days.

5.2.2. Weigh out suitable portions (e.g. 50 g or 100 g) of the paint/varnish into sterile screw cap containers and close them tightly.

5.2.3. Six non-preserved samples should be used as control samples. For this purpose, inoculate three samples with yeast (positive control) and leave the remaining three samples uninoculated (negative control or retained sample).

5.2.4. Store the samples for 4 weeks at  $40\text{ °C} \pm 2\text{ °C}$  or alternatively 18 weeks at  $30\text{ °C} \pm 2\text{ °C}$ .

5.2.5. After the 4-week ageing process, carry out the yeast load test as described in Paragraph 5.3.

## **5.3. Yeast load test**

5.3.1. Inoculate each sample (except for the negative control) with the same volume of mixed suspension equivalent to 1.0 % of the sample weight. Mix the sample thoroughly using a sterile spatula and then screw the cap onto the container in such a way that oxygen can still enter the container.



- 5.3.2. In order to determine the initial yeast load for all samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Then incubate the plates at  $30\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for a period of 3 - 7 days. Use a suitable method to then determine the number of colony-forming units.
- 5.3.3. In order to check the vitality of the yeasts being used, spread and count the mixed suspension as described under Paragraph 5.3.2.
- 5.3.4. Incubate all samples for 7 days at  $30\text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ .
- 5.3.5. In order to determine the cell count in the samples, use an appropriate dilution series and spread the samples onto nutrient agar plates or use a pour plate method. Determine the colony-forming units after incubating the plates at  $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$  for 3 - 7 days.
- 5.3.6. Repeat steps 5.3.1 to 5.3.5 at weekly intervals until at least 3 cycles have been completed. The test is concluded after a total incubation time of 6 weeks at  $30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ . Determine the final number of colony-forming units.
- 5.3.7. In order to determine the effectiveness of different biocidal concentrations depending on the incubation times, additional cell counts can be determined, e.g. 1 and 3 days after inoculation.

#### **5.4. Validity of the test**

The test is valid if the growth capacity of the inoculate can be verified on PD agar in petri dishes (see 5.1.6).

#### **5.5. Pass/Fail criteria**

- 5.5.1. The curative effect of the biocide in the paint/varnish at the added concentration is verified if the cell count is  $\leq 1000\text{ CFU/ml}$  at the end of the test.
- 5.5.2. The effectiveness of a tested biocide cannot be verified if the cell count in the biocide-free varnish after inoculation is  $< 1000\text{ CFU/ml}$  at the end of the test.

#### **5.6. Verification of a significant difference**

Use suitable statistical measures to verify any significant differences between the cell count in the samples treated with in-can preservatives and in the untreated samples.

## Annex 1 Nutrient medium

### Nutrient agar

Potato glucose agar is used to isolate, determine the number and identify yeasts and moulds.

Typical composition (g/L)

- Potato infusion 4,0
- Glucose 20,0
- Agar 15,0
- pH  $5,6 \pm 0,2$

### Preparation

Suspend 39 g of nutrient agar in 1 L of distilled water, stir until completely dissolved and then autoclave for 15 minutes at 121°C. In order to suppress the undesired growth of bacteria, add the nutrient medium chloramphenicol to produce a final concentration of 30 µg/ml.

Alternatively, sabouraud agar or malt extract agar can also be used as a nutrient medium.

## **Annex 4c**

### **Carrying out a biotest using moulds**

The applicant shall carry out a test of the product's fitness for use using the following method to determine the minimum quantity of the biocidal product<sup>5</sup> required:

#### **A laboratory method for determining the concentration of a biocidal product that is required to combat moulds in low emission and pollutant paints and varnishes according to DE-UZ 12a**

##### **1. Scope**

The method can be used to test how effective biocidal products are at preventing the growth and survival of damaging organisms in aqueous, polymer-based paints and varnishes. This method is also used for the inclusion of biocidal products in the list of approved in-can preservatives and for verifying the suitability of other mixtures.

##### **2. Health note**

National health regulations must be observed during the execution of the test. This also applies to EC Directive 200/54/EC "protection of workers from risks related to exposure to biological agents at work".

When handling paints/varnishes and biocides, the instructions in the corresponding safety data sheets and product information brochures for the safe use of the product must be observed.

##### **3. Instruments and nutrient media**

- Suitable sterile, screw cap containers (100ml);
- Sterile pipettes, nominal volume 1.0 ml and 5.0 ml;
- Sterile pipette tips;
- Sterile centrifuge beakers (35 ml);
- Sterile suction filters, size 1;
- Sterile glass or plastic petri dishes, diameter 90 or 100 mm;
- Sterile diluent; e.g. distilled water or physiological saline;
- Sterile wetting agent solution;
- Scales;
- Incubator, thermostatically controlled ( $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ );
- Autoclave;
- Drying cabinet;
- Sterile cotton swabs;

- Sterile inoculating loops or needles;
- Test tube racks;
- Test tubes;
- Test tube shaker;
- Centrifuge;
- Safety cabinet, class 2;
- Sterile nutrient media for the corresponding mould cultures, composition and production (see Annex 1);
- Mould stock cultures;
- Haemocytometer (depth 0.1 mm);
- Microscope;

#### **4. Test organisms**

The following moulds should be used for the mould load test:

##### **Moulds:**

Aspergillus oryzae	BAM 613 or NBRC 100959
Paecilomyces variotii	BAM 19 or DSM 1961 or ATCC 18502
Penicillium ochrochloron	BAM 25 or DSM 1945

Other moulds that continuously lead to infections can be additionally used in a separate inoculation suspension. It is necessary to carry out a molecular and biological characterisation of these organisms (which must be available as pure cultures) to clearly determine the strain being used. The organisms should be stored at a recognised culture collection.

#### **5. Method**

Inspect the materials received for testing before starting the test. To do this, use a sterile inoculating loop to spread the paint/varnish onto nutrient agar plates and check for any possible growth. It is necessary to check both unaged samples and also artificially aged samples (see 5.2). The Pass/Fail criteria must be met in both cases (see 5.5).

##### **5.1. Preparation of the inoculation suspension**

5.1.1. Inoculate nutrient agar plates with the test moulds approx. 14 days before they are used for the test. Sterilise the required equipment and liquids at 121°C for 20 minutes in autoclaves.

5.1.2. Fill the test tubes with 10 ml of the wetting solution under sterile conditions and

wet a cotton swab with this solution. Then wipe the spore material off the nutrient agar plates using the cotton swab and transfer it to the filled test tubes by rubbing it on the glass wall of the test tube. Use a test tube shaker to mix up the preparation.

- 5.1.3. Shake up the preparation again and then pour it through a funnel filter into a centrifuge beaker. In the case of weakly sporulating mould cultures, additional spores should be collected from other nutrient agar plates. A separate funnel filter and a separate centrifuge beaker must be used for each mould.
- 5.1.4. Centrifuge the suspensions for 10 minutes at 3,000 rpm and room temperature. Then carefully remove the supernatant.  
Wash the pellet with 10 ml of sterile, demineralised water and centrifuge the preparation again for 10 minutes at 3,000 rpm and room temperature.
- 5.1.5. Repeat the process described in 5.1.4 twice so that the spores have been washed a total of three times.
- 5.1.6. Add the pellet to 3-5 ml of sterile, demineralised water and determine the concentration of the spores using a suitable haemocytometer. (e.g. Thoma chamber, chamber depth 0.1 mm).
- 5.1.7. The spore count of the individual spore suspensions should be  **$8 \cdot 10^5 - 2 \cdot 10^6$  spores/ml**.  
Store the prepared spore suspension in a refrigerator until it is ready for use.
- 5.1.8. In order to prepare a mixed suspension, combine and mix together identical volumes of each spore suspension. The spore count for this spore suspension should also be  **$8 \cdot 10^5 - 2 \cdot 10^6$  spores/ml**.
- 5.1.9. Test the vitality of the moulds by spreading the spore suspension onto PDA plates. Incubate the plates for a maximum of 7 days at  $30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .

## **5.2. Artificial ageing**

- 5.2.1. Add the biocidal product to the paint/varnish at appropriate concentrations for the test series, vigorously mix the samples and store at room temperature for at least two days.

- 5.2.2. Weigh out suitable portions (e.g. 50 g or 100 g) of the paint/varnish into sterile screw cap containers and close them tightly.
- 5.2.3. Six non-preserved samples should be used as control samples. For this purpose, inoculate three samples with spore suspensions (positive control) and leave the remaining three samples uninoculated (negative control or retained sample).
- 5.2.4. Store the samples for 4 weeks at  $40\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$  or alternatively 18 weeks at  $30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .
- 5.2.5. After the 4-week ageing process, carry out the mould load test as described in Paragraph 5.3.

### **5.3. Mould load test**

- 5.3.1. Inoculate each sample (except for the negative control) with the same volume of spore suspension equivalent to 1.0 % of the sample weight. Distribute the spore suspension homogeneously onto the surface of the sample by carefully tilting the sample. Then screw the cap onto the container in such a way that oxygen can still enter the container.
- 5.3.2. In order to check the vitality of the mould, spread the spore suspension onto nutrient agar plates and incubate the plates for a maximum 7 days at  $30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .
- 5.3.3. Incubate all samples for 7 days at  $30 \pm 2^{\circ}\text{C}$ .
- 5.3.4. Visually inspect the surface growth of the mould.
- 0: No growth
  - 1: Weak growth (up to 10% of the surface covered)
  - 2: Medium growth (up to 30 % of the surface covered)
  - 3: Strong growth (up to 70 % of the surface covered)
  - 4: Full growth (up to 100 % of the surface covered)
- 5.3.5. Repeat steps 5.3.1 to 5.3.4 at weekly intervals until at least 3 inoculation cycles have been completed. The test is concluded after a total incubation time of 6 weeks at  $30\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .
- 5.3.6. In order to determine the effectiveness of different biocidal concentrations

depending on the incubation times, additional cell counts can be determined, e.g. 1 and 3 days after inoculation.

#### **5.4. Validity of the test**

The test is valid if the growth capacity of the mould can be verified on PD agar in petri dishes (see 5.1.9).

#### **5.5. Pass/Fail criteria**

5.5.1. The curative effect of the biocide in the paint/varnish at the added concentration is verified if no mould growth can be observed at the end of the test.

5.5.2. The effectiveness of a tested biocide cannot be verified if no mould growth in the biocide-free paint/varnish after inoculation can be verified.

#### **5.6. Verification of a significant difference**

Use suitable statistical measures to verify any significant differences between the cell count in the samples treated with in-can preservatives and in the untreated samples.

## Annex 1 Nutrient medium

### Nutrient agar

Potato glucose agar is used to isolate, determine the number and identify yeasts and moulds.

Typical composition (g/L)

- Potato infusion 4,0
- Glucose 20,0
- Agar 15,0
- pH  $5,6 \pm 0,2$

### Preparation

Suspend 39 g of nutrient agar in 1 L of distilled water, stir until completely dissolved and then autoclave for 15 minutes at 121°C. In order to suppress the undesired growth of bacteria, add the nutrient medium chloramphenicol to produce a final concentration of 30 µg/ml.

Alternatively, sabouraud agar or malt extract agar can also be used as a nutrient medium.



**Process according to the checklist for applying for the inclusion of preservatives in the list of approved in-can preservatives**

The preservative manufacturer submits an application to the German Environment Agency (UBA) in accordance with the checklist for the inclusion of the biocidal product<sup>8</sup> in the revised Basic Award Criteria DE-UZ 12a "Low-emission and low-pollutant paints and varnishes".



The UBA examines the application to ensure it is complete.



In discussion with the Federal Institute for Materials Research and Testing (BAM), the German Environment Agency recommends three graduated substance concentrations to the preservative manufacturer for the tests to assess the product's fitness for use.



The preservative manufacturer commissions a testing institution accredited for microbiological tests to carry out the tests to assess the product's fitness for use on the white wall paint (the starting formulation in Annex 1 to the checklist).



The preservative manufacturer submits the results of the tests to assess the product's fitness for use according to the checklist to BAM for evaluation. BAM sends their evaluation to the UBA.



After the evaluation has been assessed, RAL gGmbH either includes or does not include the preservative in the revised Appendix 1 (new) for the Basic Award Criteria DE-UZ 12a "Low-emission and low-pollutant paints and varnishes" and publishes the new version.

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<sup>8</sup> Product-type 6 according to the Biocidal Products Regulation (EU No. 528/2012)

## Guide formulations

### Glazes

Guide formulation for testing the effectiveness of in-can preservatives in glazes

Component, chemical	Component, name (example)	Parts by weight
	<b>*present*</b>	
Water	Water	462,5
	<b>*add slowly*</b>	
Sodium – polyacrylate, 45%	Dispex AA 4145 (BASF)	3,0
Polyether polyurethane thickener, 20%	Rheolate 212 (Elementis)	25,0
	<b>*mix*</b>	
Titan dioxide -pigment, rutile	Titan dioxide R706 (Chemours Deutschland GmbH)	200,0
	<b>*disperse completely*</b>	
Silicone-free additive, 50%	Silco Wet D-504/PEG (Silcona GmbH & Co.KG)	0,5
Silicone defoamer, >96%	Byk 024 (Byk)	2,0
Polyether siloxane copolymer emulsion approx. 24%	Tego Airex 902 w (Evonik Industries AG)	2,0
Pure acrylic dispersion, MFT<3°C	Acronal LR 9014 45% (BASF) special batch without preservation	460,0
Pigment preparation	Pigment paste pine	15,0
Water	Water	30,0
	<b>*stir for 10 min after completion*</b>	<b>1.000,0</b>

### Technical parameters

Solid content: approx. 22 %

Density: approx. 1,01 g/cm<sup>3</sup>

## White and coloured lacquers

Guide formulation for testing the effectiveness of in-can preservatives in dispersion gloss varnish

Component, chemical	Component, name (example)	Parts by weight
	<b>*present*</b>	
Water	Water	140,0
	<b>*add slowly*</b>	
Sodium – polyacrylate, 45%	Dispex AA 4145 (BASF)	6,5
Polyether polyurethane thickener, 20%	Rheolate 212 (Elementis)	17,0
Titan dioxide -pigment, rutile	Titan dioxide R706 (Chemours Deutschland GmbH)	200,0
	<b>*disperse completely*</b>	
Silicone-free additive, 50%	Silco Wet D-504/PEG (Silcona GmbH & Co.KG)	2,0
Silicone defoamer, >96%	Byk 024 (Byk)	2,0
Polyether siloxane copolymer emulsion approx. 24%	Tego Airex 902 w (Evonik Industries AG)	2,0
Pure acrylic dispersion, MFT<3°C	Acronal LR 9014 45% (BASF) special batch without preservation	580,0
Water	Water	50,5
	<b>*stir for 10 min after completion*</b>	<b>1.000,0</b>

### Technical parameters

Solid content: approx. 48 %

PVK: approx. 17 %

Density: approx. 1,21 g/cm<sup>3</sup>

Gloss, 60° >65 GE (glossy, according to EN 13300)

## Undercoats

Guide formulation for testing the effectiveness of in-can preservatives in undercoats

Component, chemical	Component, name (example)	Parts by weight
	<b>*present*</b>	
Water	Water	248,0
	<b>*add slowly*</b>	
Sodium – polyacrylate, 45%	Dispex AA 4145 (BASF)	5,0
Polyether polyurethane thickener, 20%	Rheolate 212 (Elementis)	12,0
Titan dioxide -pigment, rutile	Titan dioxide R706 (Chemours Deutschland GmbH)	70,0
Calcite	Omyacarb 2 (Omya)	200,0
Chalk	Industrie Spezial (Omya)	100,0
Clay	China Clay Polwhite (Imerys)	50,0
	<b>*disperse completely*</b>	
Silicone-free additive, 50%	Silco Wet D-504/PEG (Silcona GmbH & Co.KG)	1,0
Silicone defoamer, >96%	Byk 024 (Byk)	2,0
Polyether siloxane copolymer emulsion approx. 24%	Tego Airex 902 w (Evonik Industries AG)	2,0
Pure acrylic dispersion, MFT<3°C	Acronal LR 9014 45% (BASF) special batch without preservation	280
Water	Water	30,0
	<b>*stir for 10 min after completion*</b>	<b>1.000,0</b>

### Technical parameters

Solid content: approx. 56 %

Density: approx. 1,40 g/cm<sup>3</sup>

