**Committee for Risk Assessment**

**RAC**

Annex 2

**Response to comments document (RCOM)**

to the Opinion proposing harmonised classification and labelling at EU level of

# (1-methylethylidene)di-4,1-phenylene tetraphenyl diphosphate; aka Bisphenol A Diphosphate; aka Bisphenol A Polyphosphate

**EC number: 425-220-8**
**CAS number: 5945-33-5**

CLH-O-0000002129-76-03/A2

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**Adopted**

**28 November 2012**

**COMMENTS AND RESPONSE TO COMMENTS ON CLH: PROPOSAL AND JUSTIFICATION**

*ECHA has compiled the comments received via internet that refer to several hazard classes and entered them under each of the relevant categories/headings as comprehensive as possible. Please note that some of the comments might occur under several headings when splitting the given information is not reasonable.*

**Substance name: (1-methylethylidene)di-4,1-phenylene tetraphenyl diphosphate; aka Bisphenol A Diphosphate; aka Bisphenol A Polyphosphate**

**EC number: 425-220-8**
**CAS number: 5945-33-5**

**General comments**

| Date | Country /Organisation/MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
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| 15/12/2011 | France / MSCA | Substance identity: 20% impurities are claimed as confidential and as not affecting classification. One could expect a short explanation to guarantee that this large amount doesn't influence ecotoxicity or/and stability and that this substance should not be considered as a mixture.<br>As removal of aquatic-chronic-4 classification hangs notably on BCF results, even an effort was made to describe these data, some verification and improvements appear as necessary to make the proposal acceptable. | The typical concentration of the substance is given as 85% in IUCLID (80% - 85% concentration range) therefore the substance can be considered as a mono-constituent substance according to ECHA guidance (A mono-constituent substance is a substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).).<br>All registration dossiers for EC No. 425-220-8 available on the | We agree that the substance is a mono-constituent substance |

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| | | | ECHA website list the substance as a mono-constituent substance. The impurities listed (totaling 15% typical) are higher oligomers (typical 8 and 3%) along with one other substance (typical 4%). Information on the impurities is provided in the technical dossier and none of these impurities/components is known to have any influence on ecotoxicity. All testing conducted on this substance will have included these impurities/components in the tested sample (with slight variations in concentrations between different samples and different suppliers/manufacturers).<br>There is no evidence from the testing to suggest these have an effect on stability and effected results. | |
| 09/01/2012 | Sweden /MSCA | Data available for the current classification<br>The data set underlying the current harmonized classification of the substance is unclear. The dossier refers to the agreement reached under the former NONS scheme and the data available at that time (our understanding is that the substance, which is poorly water soluble, was assessed as not readily biodegradable, and since its Log Kow was above3 and there was no information available on whether | The harmonized classification (R53) was initially put in place based on the results showing the substance to have low water solubility, not be readily biodegradable and to have a Log Kow >3. There was acute | The clarifications are appreciated on the background of the dossier and the reasons for its submission. |

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| | | the substance was toxic at its solubility limit, the substance was classified as R53). However, in order to understand the reason for its removal, it should be made clear in the dossier on what data the classification was based. | toxicity data available on fish, Daphnia and algal, showing no toxicity at the maximum water solubility levels tested in these studies. These acute data were not sufficient to remove the R53 classification.<br>Based on this, different notifiers (i.e. Chemtura and ICL-IP) then started providing additional data to member state CA's to argue against the R53 classification. This is the data described in section 2 of the CLH report e.g. ICL-IP provided the Dutch CA (RIVM) with further Daphnia reproductions studies and Chemtura (then called Great Lakes Chemical Corporation) supplied the UK CA with bioaccumulation data on an analogous product (AFR-1) and subsequently on the substance itself (called CN-1985). Based on these results the CA's agreed to support the removal of the classification under NONS.<br><br>The different values for water solubility and log Kow come from study data conducted at | We accept the justification on the differences in water |

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| | | Data provided in the dossier<br>The data show that water solubility of the substance is low and lies between 0.415 (pH 6) and 1.88 (pH 7) and its Kow between 4.5 (pH 5.65) and >4.9 (pH 7.3). According to the dossier these differences in WS and log Kow are due to differences in substance composition. Due to its physical properties the substance can disappear from the test solution, which calls for a monitoring of actual concentration of the test solutions in order to correctly reflect the exposure concentrations. | different laboratories on test samples of the substance from different manufacturers/suppliers.<br>Therefore, some variation in results is expected.<br>The difference in water solubility (between the water solubility studies and also some of the water solubility levels recorded in ecotox testing) was an issue that was also raised by member state CA's when the original studies and arguments for removal of R53 were being discussed. This was looked into by the notifiers and CA's, but no definitive answer for the differences could be provided. It was therefore reasoned to be down to slight differences in substance composition between suppliers, plus differences between test laboratories, test methods and differences between test media used.<br><br>In all of the acute and long-term ecotox testing conducted and reported in the proposal, actual concentrations of the test solutions were measured, | solubility.<br><br>Clarification on the measured and nominal data noted. |

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| | | | so results can be expressed as measured concentrations and not just nominal concentrations.<br><br>Acute toxicity testing: For acute/short-term toxicity testing to fish, Daphnia and algal, the 'discussion' sections in the CLH report give information on the measured concentrations of test substance found during the studies.<br>In the acute studies, a condition of maximum attainable exposure is considered to have been employed.<br><br>Long-term toxicity to fish:<br>Study reference: Knight B (2003): The NOELr result of 5 mg/l is based on the initial loading rate used in this study, which used WAFs. However, measured concentration of the WAF solutions were also made, but were not included in the discussion section of this study in the CLH report. This should be updated with details from study below: | Clarification acknowledged<br><br>Clarification acknowledged |

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| | | | The early-life stage test was conducted at the following nominal loading rates of test substance (prepared as WAF): 0, 0.5, 5 mg./l.<br>Chemical analysis (HPLC) detected presence of test substance in all test samples at 0.5 and 5 mg/l. In most instances the levels were below the limit of quantification (0.0007 – 0.0019 mg/l for component peak 1 and 0.003 – 0.009 mg/l for component peak 2) of the method employed.. Quantifiable levels were obtained in Day 0 samples and in one replicate injection for component peak 1 on Day 27 for the 5 mg/l WAF.<br><br>Long-term toxicity to Daphnia:<br>Study reference: Hargreaves TL & Clayton MA (2003). The results are expressed as initial loading rates (nominal) as the study was conducted using WAFs. However, the test substance concentrations in the WAFs were also measured and the results given in the discussion section for this | Clarification acknowledged |

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| | | | study (test substance in solution was calculated to be many times less than the initial loading rates – between 0.4 and 1.9% nominal in fresh solutions and 0.3 – 1.3% in expired solutions.<br><br>The two additional Daphnia reproduction studies also had measured concentrations and the results are expressed as measured concentrations and not nominal.<br><br>All results indicate no toxicity at the limit of solubility in these studies i.e. NOEC = highest measured concentration. | Noted.<br><br>Clarification acknowledged on the measured data in the toxicity studies |
| 09/01/2012 | Portugal / Portuguese Environment Agency | Considering the present proposal, we agree with the need to establish a revised harmonised classification & labelling for Bisphenol A Polyphosphate.<br>We support the removal of the Classification and Labelling for the environment as the substance doesn't fulfil the criteria established both in CLP Regulation and 67/548/EEC Directive. | Thank you for your comments. | Support for the removal of the current classification is noted |

**Carcinogenicity - no comments received**
**Mutagenicity- no comments received**
**Toxicity to reproduction - no comments received**
**Respiratory sensitisation - no comments received**

**Other hazards and endpoints**

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| 15/12/2011 | France / MSCA | Environmental hazards¬<br><br>The key bioaccumulation test (OECD-305C; Noguchi, 1999):<br>1) OECD-305C (1981) was replaced by OECD-305 (1996), as the study is dated 1999 this is not understandable. | 1). Test method given in report as: "This test was conducted according to the "Method for Testing the Degree of Accumulation of Chemical Substances in Fish Body" stipulated in the "Testing Methods for New Chemical Substances" (July 13, 1974, Kanpogyo No, 5, Planning and Coordination Bureau. Environment Agency, Yakuhatu No 615, Pharmaceutical Affairs Burea, Ministry of Health and Welfare, and 49 Kikyoku No 392, Basic Industries Burea, Ministry of International Trade and Industry, Japan). This method is essentially the same as that in the OECD guidelines for Testing of Chemicals "Bioaccumulation: 305C, Deree of Biocenetration in Fish" (May 12, 1981).<br>There are no major differences | Clarification on the OECD 305C is acknowledged.<br>It would have been beneficial and more transparent in the explanation, if the changes in the 1996 version compared to the 1981 version were provided by the DS and why these changes are considered not relevant. |

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| | | | between the OECD-305 (1981) and the 1996 update. It is considered that a study to OECD-305 (1996) would not significantly alter the results of the current study.<br>This study has been assessed by an ecotox expert who considers the study to be valid in principle and shows that the test item does not bioaccumulate. | In principle, such an assessment should have been provided in the CLH report and available to third parties for transparent review. |
| | | 2) Even if we refer to the OECD-305C guideline, nothing is said about reporting the data to fish dry weight, so data should be reported to wet weight. | 2) On review of the study by ecotox testing department, it was considered that the data should have been reported as 'wet weight' rather than 'weight', as there is no reference in the report to indicate the fish were dried for weighing. This should be amended in the CLH report to indicate the data as wet weight. | We note the clarification on dry weight/ wet weight.<br>Please note while the CLH report submitted for public consultation (pc) will not be updated the RAC opinion will take account of the post pc information provided in the RCOM/Annex 1. |
| | | 3) As the log Kow > 4.5 or 4.9, BCF should also be expressed in relation to lipid content. | 3). The lipid content at initial exposure is given (4.1% average). From this data the BCF could be calculated to be expressed in relation to lipid content. However, on review of | We note the comments on the lipid content and agree that the BCF values would not significantly |

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| | | | the study by our ecotox testing department, it is considered that the BCF results would not alter significantly for lipid content, and that as the report shows that the test item does not bioaccumulate (no detectable test item was found in the fish so the BCF is lower than the limit of detection) no further relevant information would be obtained from expressing the BCF in relation to lipid content, as the substance is not being taken up. | change if the results were lipid normalised to 4.1%. |
| | | 4) Furthermore the reported data and calculation for "level-1" (2mg/L) and "level-2" (0.2 mg/L) are unclear. Please compile results in a single table, verify the calculations and justify the approach for separated BCF for each peak. | 4) Based on the preliminary test results for 48-hour LC50 and analytical detection limits, test concentrations of the test substance were decided as follows. The control was set as a blank test.<br>The associated IUCLID dataset for this substance has a robust study summary completed for the Noguchi 1999 study, which gives full details on the calculation of determination limits, calculation of BCFs etc These data are included in the Annex 1 to give fuller details of the results and the methods used to obtain them.<br>It was considered appropriate to | Clarification on the analytical method is noted.<br>Please note while the CLH report submitted for public consultation (pc) will not be updated the RAC opinion will include post-pc information provided in this RCOM/Annex 1. |

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| | | | calculate a separate BCF for each peak. The three peaks detected by HPLC correspond to the main 'constituent' of the substance (now regarded as the mono-constituent substance) and its related higher oligomers, based on the test item description given in the report. | We note the clarification on dry weight/ wet weight. |
| | | The second bioaccumulation study in support (OECD-305C, Hori, 1996):<br>1) It's not said if values are relative to dry or wet weight. | 1). Values should be expressed relative to wet weight. | Clarification acknowledged |
| | | 2) The preliminary acute test fixed LD50 at 500 mg/L because "no effects were observed up to the water solubility"; however when referring to table-9 solubility is rather 0.415 or 1.88 mg/L and when referring to the OECD-203 fish test in section-5.4 the measured water solubility was even 0.141 mg/L. | 2) A dispersant was used to allow preparation of a nominal concentration well above water solubility. The report does not give measured concentrations for the acute toxicity test, but in line with other studies it can be assumed the measured concentration would be significantly less than the nominal concentration of 500 mg/l. Therefore, as the report states that the 48 hr EC50 was >500 mg/l, it can be concluded that no toxicity was observed at the limit of water solubility. | Clarification acknowledged |
| | | Ecotoxicological studies:<br>1) Similarly, ecotox values cannot be superior to water solubility (e.g. NOELr with a FELS test OECD-210 cannot be equal to 5 mg/L). | 1). The NOELr result of 5 mg/l in the FELS test is based on the initial loading rate used in this study, which used WAFs. | |

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| | | | However, measured concentration of the WAF solutions were also made, but were not included in the discussion section of this study in the CLH report. This should be updated with the measured concentrations found in the study (see below).<br>The early-life stage test was conducted at the following nominal loading rates of test substance (prepared as WAF): 0, 0.5, 5 mg./l.<br>Chemical analysis (HPLC) detected presence of test substance in all test samples at 0.5 and 5 mg/l. In most instances the levels were below the limit of quantification (0.0007 – 0.0019 mg/l for component peak 1 and 0.003 – 0.009 mg/l for component peak 2) of the method employed..<br>Quantifiable levels were obtained in Day 0 samples and in one replicate injection for component peak 1 on Day 27 for the 5 mg/l WAF.<br>Therefore although the result in table refers to NOELr = 5 mg/l (which is above determined water solubility levels) the measured concentrations were | |

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| | | | below the limit of quantification of the analytical method employed (which are below the stated water solubility). It can therefore still be stated that the NOEC is equal to the maximum water solubility tested. | |
| | | 2) As the substance is poorly soluble in water but may adsorb to sediment, aquatic ecotoxicity could occur in this last aquatic sub-compartment. So, it would be expected also some discussion in the section 5.4.4 to fully argue the absence of ecotoxicity. | 2) Sediment toxicity data are not relevant for classification. However, additional information can be provided if required. | We agree that sediment toxicity is not relevant for this classification however if available this information would be beneficial as a weight of evidence |
| 07/01/2012 | Japan / Individual | As described in p.5, the substance is identified that the purity or the typical concentration is more than 80%.<br><br>On bioaccumulation estimation described at the study 1 in section 5.3..2 on p.26, the substance was analyzed as three peaks that included impurities or any analogues.<br>At the study 1 for bioaccumulation, the BCF of the Peak 3 was 159 and less at Level 2.<br>However, the purity of the substance was not stated at the study 1 and also not stated at the study 2 in section 5.3..2 on p.28. As the substance is mean 2.5% degradation after 28 day at MITI test mentioned in section 5.1..2..2 on p.24, in case that Peak 3 is originated in the impurity, the BCF of the peak 3 should not be slighted and ignored.<br>At the study 2, three test item peaks were not separated and were dealt with as one peak. The result of study 2 did not conclude the | Study 1 (Noguchi S (1999))<br>The study gives the purity of the substance as 97.4%. The three peaks observed in the HPLC relate to the main 'component' and related higher oligomers respecitvely. In the report these are given as n=1: 77.1 wt%, n=2: 17.4 wt%, n=3: 3.0wt%, which are slightly different to the typical ratio's currently given for this substance. Therefore, it was considered applicable to address BCF values for each peak i.e. for each 'component'. | Clarification acknowledged |

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| | | BCF of peak3.<br><br>Meanwhile, at Table 6 in section 1..2 on p.14, the submitter stated as remarks "Concentration range is claimed as confidential and is not provided in this public document. The value is provided in the accompanying IUCLID dossier. The confidential information does not effect the classification proposal". However, the purity is a critical element for the bioaccumulation estimation.<br><br>The substance is low purity or typical concentration as more than | Study 2 (Hori K (1996))<br>The study gives the purity of the substance as 95.0% (based on structure of main component, n=1). However, a different analytical method was used which didn't separate the substance into 3 peaks. BCF values were able to be calculated in this study as some test item was able to be detected in the fish. However, the low results are typical of a low BCF substance and basically show the test item doesn't accumulate.<br><br>Therefore, although the two bioaccumulation studies have different analytical methods, they both gives results which suggest the test item does not bioaccumulate, and hence support the argument for the removal of R53.<br><br>Substance ID in IUCLID:Typical concentration of substance is given as 85% in IUCLID for this registrants substance (80-85% concentration range) therefore the substance can be considered as a mono-constituent substance according to ECHA guidance (A mono-constituent substance is a | We note the justification on the purity of the substance and agree that the impurities do not influence ecotoxicity |

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| | | 80%, that may include any impurity up to 20%. Ingredients up to 20% can not be dealt with as the impurities. The impurities should be evaluated Human Health Hazard Assessment and Environmental Hazard Assessment as new chemical compounds. I recommend that further examination, by using the substance of well-defined purity or by evaluating the impurity as new compound, is needed to obtain conclusions for the bioaccumulation. | substance, defined by its quantitative composition, in which one main constituent is present to at least 80% (w/w).). All registration dossiers for EC No. 425-220-8 available on the ECHA website list the substance as a mono-constituent. Under NONS the substance may have been viewed as a polymer.<br>The impurities listed in IUCID for this registrants substance (totaling 15% concentration typical) are related higher oligomers of the main substance (typical 8% and 3%) along with one additional substance (typical 4%), full details are provided in the technical dossier. None of these impurities/components is known to have any influence on ecotoxicity. | |
| 09/01/2012 | Sweden / MSCA | The dossier presents data on degradation, bioaccumulation and toxicity of the substance.<br><br>Biodegradation<br>We agree that based on the data provided the substance is not readily/rapidly biodegradable.<br><br>Bioaccumulation<br>We also agree that based on the data from the two bioaccumulation tests (OECD 305 or equivalent) the substance does not meet the cut offs for bioaccumulation according to both DSD and CLP. | We agree that the testing has been conducted using a number of different procedures i.e. only at water solubility limit, over a range of concentrations and using WAFs.<br>Testing has been conducted on this substance over a period of time at a number of different laboratories and by a number of different sponsor's (registrants). | We acknowledge the clarifications provided on aquatic toxicity. |

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| | | Aquatic toxicity<br>General comments<br>The data on aquatic toxicity were produced using different procedures: (i) testing only at water solubility limit, (ii) testing at a range of concentrations, and (iii) testing using WAF method. Although in all test concentrations could not be maintained, not all tests expressed the toxicity based on measured concentrations. In general we question the applicability of WAF for toxicity testing in this particular case. We do not see this justified based on the reference to the guidance on testing of difficult substances. WAF is a method to test substances of unknown or variable composition with ingredients having different physical properties. This is not the case here since the substance is regarded as mono-constituent. In addition it has been shown that testing according to standard procedure (a range of concentration) was possible. | It is therefore realistic to expect there to be certain differences in test procedures. However, all studies have been reviewed for reliability and suitability for use, and all are considered to give valid results which can be used in the interpretation of the substances toxicity and therefore for classification purposes. The different test procedures also all produce results that are in line and consistent with each other (i.e. no toxicity at limit of water solubility, apart from minor reduction in growth in one Daphnia reproduction study – see comments below).<br>The use of WAFs in a long-term fish study and Daphnia reproduction study are considered to be applicable to this case and to give valid results.<br>Under REACH the substance is being regarded as a mono-constituent substance, as the main component (n=1) is >80% of the substance. The related higher oligomers and additional impurity are present at a maximum individual concentration of 8%, so are now considered impurities (see | |

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| | | | IUCLID dossier). However, the ratio of these 'components' has slightly varied between different notifiers substances and between different studies. Therefore, in some cases the substance was treated as having different components and therefore it was considered that WAF testing was appropriate.<br>For instance, the test item details in the long-term fish study (FELS test) are:<br>Test item consists of four components. Components 1 and 2 account for >95% of the test item on a component peak (HPLC-UV basis). The validate analytical method quantified components 1 and 2 of the test item, therefore analysis of test samples measured their components.<br>In all WAF studies conducted the concentration of test substance in the WAF solutions were measured, so results can also be expressed in measured concentrations as well as by nominal loading rates. The measured concentrations were many times less than the initial loading rates. No toxicity was observed at the highest | |

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| | | | measured concentrations, which is in line with the results of the non-WAF studies.<br>It is therefore considered that the WAF studies are applicable in the justification of the removal of the R53 classification. In addition, no other long-term fish study is available besides the WAF study so it is felt this is relevant to use.<br>It can also be argued that the WAF studies would give similar results to a 'slow-stir method' on a pure substance. | |
| | | The toxicity data presented in the dossier is summarized in table 1 (see attachment).<br><br>Conclusions<br>Based on the results from the bioaccumulation studies (showing low bioaccumulation potential of the substance) we consider that the previous reason for classification of the substance as R53 is not longer valid (if the reason was based on low water solubility of the substance, its no readily biodegradability and assumed high bioaccumulation potential based on log Kow). | The R53classification was originally based on the substance having a low water solubility, not readily biodegradable and having a high log Kow (4.5, >4.9, leading to assumed high potential bioaccumulation). Therefore, based on the results of bioaccumulation studies we agree that R53 is no longer valid. | Support for the removal of the current classification is noted |
| | | The dossier submitter provided also toxicity data as an additional evidence to prove that the substance is not toxic at its water solubility limit. In our view these results should be further explained end discussed (see the table above) in order to allow such a conclusion.<br>According to the classification criteria based on chronic toxicity data | It is our opinion that the provided ecotox data (acute and long-term) supports the removal of R53. However, the results could be further explained and expanded in the CLH report to | Clarification on the toxicity studies noted. The measured water solubility ranges between 0.415 |

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<td>introduced by ATP 2, these unclear results may justify Chronic II classification based on the fact that the substance is not readily biodegradable and that the chronic adverse effects were measured at its water solubility limit (which is between 0.415 (pH 6) and 1.88 (pH 7)).<br><br>ECHA comment: The attached document 'Supporting document to SE Comments on Annex XV dossiers proposing harmonised Classification.docx' is copied below.<br><br>
<table>
<tr><th>Trophic level</th><th>Acute toxicity</th><th>Chronic toxicity</th><th>Comments</th></tr>
<tr><td>Fish</td><td>OECD 203<br>No effects at 0.141 mg/l (water solubility limit in the test)</td><td>OECD 210<br>FELS<br>WAF method</td><td></td></tr>
<tr><td>Invertebrates</td><td>OECD 202<br>No effects at 0.195 mg/l (water solubility limit in the test)</td><td>OECD 211<br>Three test available: (i)WAF, (ii) two testing a number of concentrations;<br>NOEC (growth) = 1.2 mg/l in Desjardin</td><td>Although the significant reduction in growth was not seen at similar concentration in Desjardin at al (2002a), the occurrence of this effect in Desjardin at al (2002b), should be</td></tr>
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<td>clarify the argument for R53 removal (arguments summarised below).<br><br>Acute fish: No toxicity at limit of solubility (maximum 0.141 mg/l) in study.<br><br>Acute Daphnia: No toxicity at limit of solubility (maximum 0.195 mg/l) in study/<br><br>Acute algal: No toxicity at limit of solubility (no measured concentrations – see below).<br><br>The measured concentrations of test substance in unfiltered samples of the test culture, ranged between 1.61 and 2.93 mg/l, with an overall mean measured level of 2.17 mg/l. No test material was detected (<20 µg/l) in filtered samples of medium. These data were not unexpected in view of the low aqueous solubility of the test material. Although the concentration of dissolved test substance to which the algae were exposed was not identified, a condition of maximum attainable exposure is considered</td>
<td>and 1.88 mg/L. Classification as Chronic 2 for non-rapidly degradable substances for which chronic data is available requires a NOEC or ECx of >0.1 and < 1 mg/L. According to the 2nd ATP Chronic 4 classification is applied unless there is other evidence such as a chronic toxicity NOEC > water solubility or > 1 mgl/L. The lowest NOEC from the 3 provided studies is 1.2 mg/L. In addition acute toxicity values show no effects at the solubility level.<br><br>Clarification acknowledged</td>
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<tr><td></td><td></td><td>et al (2002b)</td><td>discussed since it was measured within the range of wate r solubility and it is therefore considered valid for the classification.</td></tr>
<tr><td>Algae/Aquatic plants</td><td>OECD 201 (96 hr) No effects at water solubility limit (no actual concentration measured)</td><td></td><td>It is not stated why the exposure time of 96hr instead of 72 hr was chosen. In addition the real exposure concentration is not known.</td></tr>
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</td><td>to have been employed.<br><br>After 96 hours, analysis of medium containing CN-1985 which had been incubated without algal cells gave similar results to test medium incubated in the presence of algal cells (1.77 mg/l compared to 1.61 mg/l); this indicates that the presence of algal cells had not affected the stability of the test substance.<br><br>There is no data to indicate why a 96 hr exposure period was used rather than 72 hour. One possibility is that the study, as well as being conducted to meet OECD Guideline 201 and EU Method C.3, was also conducted to meet US.EPA TSCA Environmental Effects Testing Guidelines, 40 CFR, Part 797.1060 "Freshwater Algae Acute Toxicity Test". Studies conducted for EPA guidelines may have longer exposure periods than standard OECD methods.<br><br>However, the use of a 96 hour exposure does not have an impact on these results on</td><td>Clarification acknowledged<br><br>Clarification acknowledged and we agree that the extended exposure period does not affect the study results.</td></tr>
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| | | | review of the study, and in fact could be argued to give more reliable data than a 72 hour exposure period. The results of this study would not be altered by using either 72 h or 96 h data i.e. no toxicity at solubility limit at either time endpoint.<br><br>Long-term fish:<br>OECD 201 FELs method, WAFs used.<br>The use of WAF is considered applicable for this substance.<br>The NOELr result of 5 mg/l in the FELS test is based on the initial loading rate used in this study, which used WAFs. However, measured concentration of the WAF solutions were also made, but were not included in the discussion section of this study in the CLH report. Additional details from on the measured concentrations found in the study are provided (see below).<br>The early-life stage test was conducted at the following nominal loading rates of test substance (prepared as WAF): 0, 0.5, 5 mg./l.<br>Chemical analysis (HPLC) detected presence of test substance in all test samples at | Clarification acknowledged |

| Date | Country / Organisation/ MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
|---|---|---|---|---|
| | | | 0.5 and 5 mg/l. In most instances the levels were below the limit of quantification (0.0007 – 0.0019 mg/l for component peak 1 and 0.003 – 0.009 mg/l for component peak 2) of the method employed.. Quantifiable levels were obtained in Day 0 samples and in one replicate injection for component peak 1 on Day 27 for the 5 mg/l WAF.<br>Therefore although the result in the table refers to NOELr = 5 mg/l (which is above determined water solubility levels) the measured concentrations were below the limit of quantification of the analytical method employed (which are below the stated water solubility). It can therefore still be stated that the NOEC is equal to the maximum water solubility tested, which supports removal of R53, as no effects were observed at the limit of water solubility.<br><br>Long-term Daphnia:<br>Study reference: Hargreaves TL & Clayton MA (2003). The results (EC50 >5 ppm and NOELR 5 ppm) are expressed as initial loading rates (nominal) as the | Clarification acknowledged |

| Date | Country / Organisation/ MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
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| | | | study was conducted using WAFs. However, the test substance concentrations in the WAFs were also measured and the results given in the discussion section for this study (test substance in solution was calculated to be many times less than the initial loading rates – between 0.4 and 1.9% nominal in fresh solutions and 0.3 – 1.3% in expired solutions.<br>This supports removal of R53 as no effects observed at limit of water solubility in study.<br><br>Study reference: Desjardin, D et al (2002 a): The results are expressed as the measured concentration of test substance rather than nominal and therefore reflect the maximum water solubility tested. The highest nominal test concentration was 3.0 mg/l, which lead to maximum mean measured concentration of 1.8 mg/l (61% of nominal). The mean measured concentrations were used to express test results.<br><br>Daphnia magna exposed to the test substance up to a concentration of 1.8 mg/l for 21 | Clarification acknowledged<br><br>Clarification acknowledged and |

| Date | Country / Organisation/ MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
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| | | | days showed no significant reductions in survival, reproduction or growth. Consequently, the no mortality/immobility concentration and NOEC were 1.8 mg/l, and the LOEC was >1.8 mg/l. The MATC (maximum acceptable toxicant concentration) was determined to be >1.8 mg/l. The 21-day EC50 was estimated to be >1.8 mg/l.<br><br>This supports the removal of R53, as no toxicity was observed at the maximum water solubility tested (NOEC = water solubility) and the NOEC is >1 mg/l.<br><br>Study reference: Desjardin, D et al (2002 b): The results are expressed as the measured concentration of test substance rather than nominal and therefore reflect the maximum water solubility tested. The highest nominal test concentration was 3.0 mg/l, which lead to maximum mean measured concentration of 1.4mg/l (45% of nominal). The mean measured concentrations were used to express test | appreciated as this study is the most relevant for the removal of the classification<br><br>Noted<br><br>Clarification acknowledged |

| Date | Country / Organisation/ MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
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| | | | results.<br><br>Daphnia magna exposed to the test substance up to a concentration of 1.4 mg/l for 21 days showed no significant reductions in reproduction or survival. However, a treatment related reduction in growth was apparent in the highest treatment level (1.4 mg/l). Consequently, the no mortality/immobility concentration and NOEC were 1.2 mg/l, and the LOEC was 1.4 mg/l. The MATC (maximum acceptable toxicant concentration) was determined to be 1.3 mg/l. The 21-day EC50 was estimated to be >1.4 mg/l.<br><br>Although a minor reduction in growth was seen in the highest treatment level (1.4 mg/l) it is still considered that this data can be used to support the removal of R53. The effect at 1.4 mg/l was only a reduction in growth. Survival and reproduction were not affected at the 1.4 mg/l exposure level and lead to the mortality/immobility NOEC to be 1.4 mg/lm the 21-day EC50 to be >1.4 mg/l and the NOEC for | Clarification noted however some further specification on this minor reduction such as actual values would have been appreciated in the CLH report |

| Date | Country / Organisation/ MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
|---|---|---|---|---|
| | | | reproduction to be 1.4 mg/l.<br><br>The difference in growth between the pooled group and the 1.4 mg/l treatment group was statistically significant, but the difference was very slight (giving a NOEC of 1.2 mg/l).<br><br>As this affect was only very slight and a similar affect was not observed at higher measured concentration (1.9 mg/l) in the Desjardin, D et al (2002 a), it is argued that the removal of R53 is still valid. In addition, the NOEC is still >1 mg/l so should be suitable to remove the R53 classification.<br><br>We do not agree that the results may justify chronic II classification, as although there is a chronic NOEC below measured water solubility (NOEC 1.2 mg/l, maximum water solubility in study 1.4 mg/l) , the associated EC50 is greater than the measured water solubility in the study. | Clarification noted however some further specification on the significant difference, such as actual values would have been appreciated in the CLH report<br><br>Noted<br><br>Noted |
| 09/01/2012 | Finland / MSCA | Environment:<br>We have concerns on the relevance, adequacy and validity of the BCF tests presented in the CHL Report:<br>- Acetone in not allowed to be used as a solvent in the OECD 305 | Our ecotox testing department argue that acetone is a standard solvent used in ecotox testing and should be appropriate for | While the use of dispersants is not recommended in the revised |

| Date | Country / Organisation/ MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
|---|---|---|---|---|
| | | guideline<br>- It is not clear what the three peaks in test 1 mean, is there some indication of the substance although the determination limit is not reached or are the peak values only based on nominal values in the test water.<br>- It is said in the text that there is no detectable test item found in the fish in test 1 which could mean that the test is not valid for this substance and a test using oral uptake should be performed.<br>- In test 2 some test item was being seen in the fish but were they used in BCF calculations<br>- How is the determination limit determined in the tests.<br>- Why are the determination limits different in different peaks in test 1 and what are they based on.<br>- How is the equilibrium determined in the tests.<br>- It would not be convincing to base 'no classification' on a test that does not find the substance in water or in the fish**.** If this is not the case and the validity of the tests are in fact good there should be more information in the CLH Report. | OECD 305, and does not affect the results of the current bioaccumulation studies.<br>-. The three peaks observed in the HPLC relate to the main 'component' and related higher oligomers. In the report these are given as n=1: 77.1 wt%, n=2: 17.4 wt%, n=3: 3.0wt%, which are slightly different to the typical ratio's currently given for this substance. Therefore, it was considered applicable to address BCF values for each peak i.e. for each 'component'.<br>-. The lack of detectable test item found in test 1 (Noguchi) is not considered to be due to an 'invalid' study, rather down to the nature of the test substance and the low limits of detection and quantification available from the analytical methods. In view of other data (other bioaccumulation study and chronic studies in fish and Daphnia) it is considered that an oral uptake study should not be required for the justification of R53 removal.<br>- The Annex 1 includes full details on the calculation of determination limits (for both bioaccumulation studies).. | versions of the OECD 305, the original OECD 305C, as used in the report does allow for the use of acetone as a dispersant<br><br>Clarification on the HPLC noted, |

| Date | Country / Organisation/ MSCA | Comment | Dossier submitter's response to comment | RAC's response to comment |
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| | | | -. It can be concluded from the BCF results, which show in test 1 that no detectable test item was found and in test 2 that minimal test item was found, that equilibrium has been achieved in these studies. | An updated CLH report has not been submitted |

**ATTACHMENTS RECEIVED:**

1. **Supporting document to SE Comments on Annex XV dossiers proposing harmonised Classification.docx**. Submitted by Sweden/ MSCA. Comment is copied in the table.

**Annex I – Additional clarification regarding bioaccumulation data**

**CLH Proposal on (1-methylethylidene)di-4,1-phenylene tetraphenyl diphosphate (CAS No. 594503305, EC No. 425-220-8)**
**Re: Additional information provided in response to public consultation and request from RAC Rapporteur**
Background: As the CLH report has not been amended following the public consultation, additional clarification regarding the bioaccumulation data is provided in this annex.
The additional data below mainly relates to the two bioaccumulation studies that were included in the CLH report.:
Key study: Noguchi S (1999)
Supporting study: Hori K (1996)

The comments from the public consultation raised some specific questions regarding the two bioaccumulation studies, which were answered in the responses to the public consultation e.g. it was confirmed that BCF results should be expressed as wet weight rather than dry weight etc.
However, it was stated that further information on these studies would be provided to address the more general requests from the public consultation for additional data on these studies. These data are summarised below.

**Bioaccumulation Data:**
Two bioaccumulation studies were addressed in the CLH report:

Key study: Noguchi S (1999)
Supporting study: Hori K (1996)

The associated IUCLID dossier on this substance contains full robust study summaries on both these studies, which fully cover all the methods, analytical techniques, calculations, results etc. These robust study summaries have more information on the studies than are described in the CLH report and can therefore be used to cover all the questions raised on these studies from the public consultation.

The key points which were raised in the public consultation and addressed in the IUCLID robust study summaries are addressed below.

**Noguchi S (1999):**
Further information was requested to justify the calculations used and to justify the approach for separated BCF for each peak.

**Noguchi S (1999): Details on Analytical Methods and BCF Calculation**

Test concentrations:
Based on preliminary test results for the 48-hour LC50 and analytical detection limits, test concentrations of the test substance were decided on as follows. The control was set as a blank test.
Level 1: 2 mg/l (as the test substance)
Level 2: 0.2 mg/l (as the test substance).

*4.7 Analysis of test water and fish:*
Three peaks were detected by high-performance liquid chromatography (HPLC) analysis of the test substance. The peaks of the chromatogram were named peak 1, peak 2 and peak 3, respectively, in elution order. The concentration of each peak was described using the concentration shown in section 4.7.3(2) without consideration of the constituent ratio.

*4.7.1 Frequency of analysis:*
The concentrations of the test substance in the test water for both level 1 (2 mg/l) and 2 (0.2 mg/l) were analysed 16 times, twice per week for eight weeks (n=1). The concentrations in the test fish for both levels were analysed 4 times; in weeks 2, 4, 6 and 8 (n=2). The control fish were analysed at the initiation and the termination of exposure (n=2).

*4.7.2 Pretreatment for analysis:*
(1) Test water:
Aliquots of the test water were taken from each test tank. The sample volumes were 25 ml for level 1 and 250 ml for level 2.
The samples were prepared for HPLC analysis as follows:

Test water
- Added test water for recovery test, 225 ml (graduated cylinder) (only level 1)

- Column chromatography*

↓

Elute

- Filled up to 5 ml (water, volumetric flask)

↓

Sample for HPLC analysis

*Conditions of column chromatography
Sep pak $C_{18}$
Conditionings: Acetonitrile 10 ml
Water 10 ml
Loading: Whole volume of the solution was loaded
Elution: Acetonitrile 3 ml

(2) Test Fish:
Test fish were taken from each test tank and prepared for HPLC analysis as follows:

Test fish:

- Measurement of weight and body length
- Chopped into pieces
- Added acetonitrile 90 ml (graduated cylinder)
- Homogenization (polytron, ca. 1 min)
- Washed with 20 ml of acetonitrile
- Centrifugation (7000 x g, 5 min)

↓

Supernatant → Residue

- Filtration (adsorbent cotton)
- Filled up to 150 ml (acetonitrile, volumetric flask)
- Removed 5 ml (transfer pipet)

- Added ion-exchanged water 150 ml (graduated cylinder)
- Added sodium chloride, 45 g (even balance)
- Added ethyl acetate 50 ml (graduated cylinder)
- Shaken (5 min)

↓

Ethyl acetate layer ⟶ Water layer

- Filtration and dehydration (IPS filter paper)
- Evaporation to dryness (rotary evaporator, ca. 40ºC, purge by nitrogen)
- Added acetonitrile 3 ml (transfer pipet)
- Irradiation with supersonic waves (ca 30 sec)
- Filled up to 5 ml (water, volumetric flask)

Sample for HPLC analysis

*4.7.3 Quantitive analysis for test substance and metabolite:*
The samples for HPLC analysis in pretreatment were analysed under the following analytical conditions. The concentration of the test substance in the sample for HPLC analysis solutions was determined based on a comparison of the peak area on the chromatogram of the sample solution with that of a standard solution.

(1) Analytical conditions:
Instrument: High-performance liquid chromatograph
Pump: Shimadzu Corporation, type LC-6A
Detector: Shimadzu Corporation, type SPD-6AV
Column: L-column ODS; 15 cm x 4.6 mm I.D, stainless steel
Column temperature: 30ºC
Eluent: A:acetonitrile B:water

Gradient Conditions

| | Time (min) | A (ml) | B (ml) |
|---|---|---|---|
| Analysis of test water | 0 | 0.95 | 0.05 |
| | 5 | 1.0 | 0 |
| | 15 | 1.0 | 0 |
| Analysis of test fish | 0 | 0.85 | 0.15 |
| | 10 | 1.0 | 0 |
| | 20 | 1.0 | 0 |

Flow rate: 1 ml/min

Measurement wavelength: 260 nm
Sample size: 100 µl
Detector output: 0 8 V/AU

(2) Preparation of standard solution:
The standard solution to determine the concentration of the test substance in the sample solutions was prepared as follows – 100 mg of the substance supplied was dissolved in acetonitrile to obtain 1000 mg/L solution. 10 mg/L standard solution was then prepared from this solution by dilution with acetonitrile/water (3/3 V/V).

(3) Calibration curve:
5.0, 10 and 20 mg/l standard solutions were prepared by the same method as described in (2) (Preparation of standard solution). These solutions were analyzed according to the analytical conditions described in (1) (Analytical conditions). A calibration curve was constructed based on the relationship between the peak area on the chromatograms and the respective concentrations.
In consideration of the background interference, the lowest detectable peak area of the test substance was regarded as follows, which corresponded to the following concentrations:
Peak 1: For analysis of test water = 1400 µV/sec (Test substance 0.14 mg/l)
Peak 1: For analysis of test fish = 3000 µV/sec (Test substance 0.32 mg/l)
Peak 2: For analysis of test water = 1400 µV/sec (Test substance 0.81 mg/l)
Peak 2: For analysis of test fish = 1400 µV/sec (Test substance 0.82 mg/l)
Peak 3: For analysis of test water = 1400 µV/sec (Test substance 4.8 mg/l)
Peak 3: For analysis of test fish = 1400 µV/sec (Test substance 4.8 mg/l)

*4.7.4 Recovery and blank test:*
(1) Method
A specified amount of the test substance was spiked to test water and fish homogenate for the recovery test, followed by pretreatment (described in 4.7.2, pretreatment for analysis). Blank tests were also conducted in the same manner, only without the test substance. All the recovery and blank tests were performed in duplicate.

(2) Results of recovery test
In the blank test, the chromatogram of HPLC had no peaks interfering with determination of the test substance concentration. The duplicate recovery rates and the average of them in the pretreatment are shown below.
For analysis of spiked water (50 µg test substance spiked)

| | | | |
|---|---|---|---|
| Peak 1 | 92.8% | 90.8% | Average: 91.8% |
| Peak 2 | 88.9% | 90.7% | Average: 89.8% |
| Peak 3 | 90.4% | 90.0% | Average: 90.2% |

For analysis of spiked fish homogenates (1500 µg test substance spiked)

| | | | |
|---|---|---|---|
| Peak 1 | 79.5% | 83.8% | Average: 81.6% |
| Peak 2 | 83.6% | 82.3% | Average: 82.9% |
| Peak 3 | 83.8% | 82.7% | Average: 83.2% |

The average recovery rates were used as correction factors for the determination of the test substance concentrations in the analytical samples.

*4.7.5 Calculation of the test substance concentration in sample and minimum limit of determination*
(1) Calculation of the test substance concentration in test water:
The equation below was used to obtain the concentrations.

I = P x (A(t) / A(std)) / B x C / F x 100 / H
Where
I: Concentration of test substance in test water (mg/L)
A: Peak area (µV/sec); A(std): Standard solution; A(t): Sample
B: Ration of portion used for analysis
C: Final volume
F: Recovery rate
H: Volume of test water taken out
P: Concentration of standard solution

(2) Determination limit of the test substance in test water:
The determination limit** was calculated based on that in 4.7.3 (3) (quantitative analysis for test substance and metabolite – calibration curve) as follows:

| | | |
|---|---|---|
| Level 1 | Peak 1 | 0.031 mg/l |
| | Peak 2 | 0.18 mg/l |
| | Peak 3 | 1.1 mg/l |
| Level 2 | Peak 1 | 0.0031 mg/l |
| | Peak 2 | 0.018 mg/l |
| | Peak 3 | 0.11 mg/l |

** Minimum determination limit of the test substance (mg/L or µg/g)
A / (B/100) x (C x E / D)
Where:
A: Minimum determination limit of the test substance on the calibration curve (mg/l)
B: Recovery rate (%)

C: Sampling volume of test water (ml) or weight of fish (g)
D: Final volume of sample solution (ml)
E: Ratio of he portion, used for analysis to whole volume.

*4.8 Calculation of bioconcentration factors (BCFs)*
BCFs were calculated using the equations below.

K: Concentration of test substance in test fish (µg/g)
K = {P x (A(t) / A(std)} / (B x D x C / G – E) / F x 100
Where:
A: Peak area (µV/sec); A(std): Standard solution; A(t): Sample
B: Ratio of portion used for analysis
C: Final volume
D: Dilution factor
E: Average concentration of blank in analysis of control
F: Recovery rate
G: Weight of test fish (g)

BCF (J) = K / H
Where:
K: Concentration of test substance in test fish (µg/g)
H: Average concentration of test substance in test water (mg/l)

From the minimum determination limit of the test substance in 4.7.5 (*Calculation of the test substance concentration in sample and minimum limit of determination),* BCFs could be obtained for cases of a BCF exceeding the following:

| | | |
|---|---|---|
| Level 1 | Peak 1 | 1.1 |
| | Peak 2 | 2.7 |
| | Peak 3 | 16 |
| Level 2 | Peak 1 | 11 |
| | Peak 2 | 27 |
| | Peak 3 | 159 |

*5 Factors possibly affecting accuracy*
No adverse effects on the reliability of this test were noted.

**Results**

*6.1 Concentrations of test substance in test water*
The measured concentrations of the test substance in test water are shown below (Table 1):
Each average concentration of the test substance in test water was maintained at larger than 90% or more of the nominal concentration.

**Table 1: Measured concentrations of the test substance in test water (average value at times from start of exposure). (units: mg/l)**

| Peak | Level | 2 weeks | 4 weeks | 6 weeks | 8 weeks |
|---|---|---|---|---|---|
| 1 | 1 | 1.89 | 1.87 | 1.84 | 1.84 |
| | 2 | 0.180 | 0.180 | 0.181 | 0.181 |
| 2 | 1 | 1.92 | 1.88 | 1.85 | 1.84 |
| | 2 | 0.182 | 0.182 | 0.182 | 0.182 |
| 3 | 1 | 1.92 | 1.89 | 1.85 | 1.85 |
| | 2 | 0.182 | 0.183 | 0.183 | 0.183 |

*6.2 Bioconcentration factors*
BCFs are shown below (Table 2).
The ranges of BCF for the test substance were as follows:

| | Level 1 (2 mg/l) | Level 2 (0.2 mg/l) |
|---|---|---|
| Peak 1 | ≤ 1.1 -1.2 | ≤ 11 |
| Peak 2 | ≤ 2.7 | ≤ 27 |
| Peak 3 | ≤ 16 | ≤ 159 |

**Table 2: BCFs**

| Peak | Level | 2 weeks | 4 weeks | 6 weeks | 8 weeks |
|---|---|---|---|---|---|
| 1 | 1 | ≤ 1.1<br>≤ 1.1 | ≤ 1.1<br>≤ 1.1 | ≤ 1.1<br>1.2 | ≤ 1.1<br>≤ 1.1 |
| | 2 | ≤ 11<br>≤ 11 | ≤ 11<br>≤ 11 | ≤ 11<br>≤ 11 | ≤ 11<br>≤ 11 |
| 2 | 1 | ≤ 2.7<br>≤ 2.7 | ≤ 2.7<br>≤ 2.7 | ≤ 2.7<br>≤ 2.7 | ≤ 2.7<br>≤ 2.7 |
| | 2 | ≤ 27<br>≤ 27 | ≤ 27<br>≤ 27 | ≤ 27<br>≤ 27 | ≤ 27<br>≤ 27 |
| 3 | 1 | ≤ 16 | ≤ 16 | ≤ 16 | ≤ 16 |

| | | | | | |
|---|---|---|---|---|---|
| | | ≤ 16 | ≤ 16 | ≤ 16 | ≤ 16 |
| | 2 | ≤ 159<br>≤ 159 | ≤ 159<br>≤ 159 | ≤ 159<br>≤ 159 | ≤ 159<br>≤ 159 |

*6.3 Results of test fish observation*
No abnormality in behaviour or appearance was noted.

This information fully describes the test methods and calculation methods and therefore confirm that the BCF results reported are valid and suitable for use in the proposal.

**Hori K (1996):**
A different analytical method was used to the key study. The method used is fully described below.

**Hori K (1996): Details on Analytical Methods and BCF Calculation**

*13.5 Test concentrations:*
Considering the results of the preliminary test for 48-hrs $LC_{50}$ and analytical detection limits of the test substance, the test concentrations of the test substance were nominated as follows. Control as blank test was also provided.
Level 1: 1.0 mg/l
Level 2: 0.1 mg/l

*13.6 Analysis of test water and test fish*
As the test substance could not be determined by instrumental analysis, the phenol which was produced by alkaline hydrolysis reaction was analysed by high performance liquid chromatography. It was assumed that a peak on the chromatogram was that of the test substance, and displayed the concentration of the substance as the concentration shown in 13.6.3 (2) (preparation of standard solution).

*13.6.1 Frequency of analysis*
Test water analysis: Twice a week
Test fish analysis: Every two weeks ($2^{nd}$, $4^{th}$, $6^{th}$ and $8^{th}$ week)
Control fish analysis: Before the initation and the termination of exposure

*13.6.2 Pretreatment for analysis*

(1) Test water:
An aliquot of the test water was taken from each test tank (Level 1 20 mL and level 2 200 ml) and then pretreated to prepare samples for high performance liquid chromatography analysis as follows:

Test water (level 1 20 mL, level 2 200 ml)
←Dilution water 180 mL (graduated cylinder) (level 1)
←Sodium chloride, 60 g (even balance)
←Dichoromethane 50 m (graduated cylinder)
- Shake (ca. 10 min)

↓

Under layer → Upper layer
- Filtrate (1PS filter paper)
- Dry up (ca. 40°C, rotary evaporator, nitrogen purge)

← Ethanol, 5 ml (volumetric pipet)
← Purified water, 5 ml (volumetric pipet)
← Potassium hydroxide, 1.0 g (even balance)
- Heat (reflux, ca. 100°C, ca. 1.5 hour)
- Cool (allow to stand, ca. 30 min)

← Hydrochloric acid, 2 ml (volumetic pipet)
- Fill up to 20 ml (ourified water, volumetric flask)

↓

Sample for HPLC analysis

(2) Test fish

Test fish were taken from each test tank and then pretreated to prepare samples for HPLC analysis as follows:

Test fish
- Measure weight and body length
- Chop into pieces

← Acetonitrile, 100 ml (graduated cylinder)
- Homogenize (polytron, ca. 1 min)
- Centrifuge (7000 x g, 5 min)

↓

Supernatant → Residue

- Filtrate (glass wool)
- Fill up to 150 ml (acetonitrile, volumetric flask)
- Take out 10 ml (transfer pipette)
- ← Sodium chloride, 60 g (even balance)
- ← Purified water, 200 ml (graduated cylinder)
- ← Dichloromethane, 50 ml (graduated cylinder)
- Shake (ca. 10 min)

↓

Under layer → Upper layer

- Filtrate (glass wool)
- Dry up (ca. 40°C, rotary evaporator, nitrogen purge)
- ← Ethanol, 5 ml (volumetric pipette)
- ← Purified water, 5 mL (volumetric pipette)
- ← Potassium hydroxide, 1.0 g (even balance)
- Heat (reflux, ca. 100°C, ca. 1.5 hour)
- Cool (allow to stand, ca. 30 min)
- ← Hydrochloric acid, 2 ml (volumetric pipette)
- Fill up to 20 mL (purified water, volumetric flask)

↓

Sample for HPLC analysis

*13.6.3 Quantitative analysis*

The samples for HPLC analysis described in 13.6.2 (*Pretreatment for analysis)* were analysed with HPLC under the following conditions. The concentration of the test substance in the finally diluted solution was proportionally calculated by comparing a peak on the chromatogram of the sample solution with that of a solution of known concentration (see Tables 4, 5, Fig.6 and Tables 7, 8, 9, Figs. 8, 9, 10.

(1) Analytical conditions:
Instrument: High performance liquid chromatograph
Pump: Hitachi Co., Ltd. Type L-6000

Detector: Japan Spectroscopic Co., Ltd. Type 821-FP
Column: L-column ODS, 15 cm x 4.6 mm (stainless steel)
Eluent: Acetonitrile/purified water (1/1 V/V)
Flow rate: 1.0 ml
Wave length: Excitation wave 275 nm, emission wave 584 nm
Sample size: 20 µl
Sensitivity: Detector: 0.4 V/FS, Recorder: Range 10 mV

(2) Preparation of standard solution:
Standard solution for HPLC analysis was prepared as follows. 0.1 g of the test substance precisely weighed was dissolved in methanol to obtain 1000 mg/L of the test substance solution. 200 mg/L of the test substance solution was then prepared by diluting it with methanol. 1.0 mg/L of standard solution was prepared as follows.

Test substance, 20 µg (200 mg/L x 100 µl)
← Ethanol, 5 mL (volumetric pipette)
← Purified water, 5 ml (volumetric pipette)
← Potassium hydroxide, 1.0 g (even balance)
- Heat (reflux, ca.100°C, ca 1.5 hour)
- Cool (allow to stand, ca. 30 min)

← Hydrochloric acid, 2 ml (volumetric pipette)
- Fill up to 20 mL (purified water, volumetric flask)

↓
1.0 mg/l of standard solution

(3) Calibration curve:
0.5, 1.0 and 2.0 mg/l of standard solutions, which were prepared by the method mentioned in (2) (*Preparation of standard solution,)* were analysed according to the analytical conditions described in (1) (*Analytical conditions*). A calibration curve was drawn based on the relation between the peak area on the chromatograms and the respective concentrations.
The lowest detectable peal area was regarded as 1500 µV/sec, considering the noise level, which corresponded to 0.049 mg/l of the test substance.

*13.6.4. Recovery and blank test*

(1) Method:
A known amount of the test substance was added into test water and fish homogenate, respectively, and pretreated in accordance with the method described in 13.6.2 (*Pretreatment for analysis*), then analysed. A blank test was also performed in exactly the same way without the test substance. These tests were carried out in duplicate. As the result of the blank test, the chromatogram of HPLC had no peak to interfere

with the determination of concentration of the test substance. The recovery rate employed in this test was an average of the measured values. The recovery rates were used as a correction factor, for the determination of the test substance in the analytical samples.

(2) Recovery rate:

Analysis of test water (20 µg of the test substance was added)
Duplicates: 95.1% and 96.0%
Average: 95.6%

Analysis of test fish (300 µg of the test substance was added)
Duplicates: 94.2% and 91.3%
Average: 92.8%

*13.6.5 Calculation of concentration of test substance in analytical sample and minimum limit of determination:*

(1) Calculation of concentration of test substance in test water:
The equation below was used to obtain the concentrations.

I = P x (A(t) / A(std)) / B x C / F x 100 / H
Where
I: Concentration of test substance in test water (mg/l)
A: Peak area (µV/sec); A(std): Standard solution; A(t): Sample
B: Ration of portion used for analysis
C: Final volume
F: Recovery rate
H: Volume of test water taken out
P: Concentration of standard solution

(2) Minimum limit of determination of test substance in test water
The minimum limit of detection* were calculated as on the basis of the minimum limit of detection described in 13.6.3 (*Quantitative analysis – calibration curve).*

Level 1: 0.051 mg/l
Level 2: 0.0051 mg/l

* Minimum determination limit of the test substance (mg/L or µg/g)
A / (B/100) x (C x E / D)

Where:
A: Minimum determination limit of the test substance on the calibration curve (mg/l)
B: Recovery rate (%)
C: Sampling volume of test water (ml) or weight of fish (g)
D: Final volume of sample solution (ml)
E: Ratio of the portion, used for analysis to whole volume.

(3) Determination of concentration of test substance in test fish
Calculated by the equations below:

K: Concentration of test substance in test fish (μg/g)
K = {P x (A(t) / A(std)} / (B x D x C / G – E) / F x 100
Where:
A: Peak area (μV/sec); A(std): Standard solution; A(t): Sample
B: Ratio of portion used for analysis
C: Final volume
D: Dilution factor
E: Average concentration of blank in analysis of control
F: Recovery rate
G: Weight of test fish (g)

BCF (J) = K / H
Where:
K: Concentration of test substance in test fish (μg/g)
H: Average concentration of test substance in test water (mg/l)

(4) Minimum limit of determination of test substance in test fish
The minimum limit of determination was calculated on the basis of the minimum limit of detection described in 13.6.3 (3) (*Quantitative analysis – calibration curve).* It was calculated to be 0.53 μg/g when fish weight was assumed as 30 g.*13.7 Calculation of bioconcentration factor (BCF)*

BCFs were calculated as described in Determination of concentration of test substance in test fish.
From the minimum limit of detection of the test substance in the test fish obtained (0.53 μg/g), it is possible that the calculation of BCF is provided when the BCF is higher than the following figures:
Level 1: 0.6
Level 2: 5.7

***14 Results***

*14.1 Concentration of test substance in test water*
The measured concentrations of the test substance in test water are shown in Table 1 (below):
**Table 1: Measured concentrations of the test substance in test water (average value at the time elapsed from starting of exposure) (Unit: mg/l)**

| | 2 weeks | 4 weeks | 6 weeks | 8 weeks |
|---|---|---|---|---|
| Level 1 | 0.979 | 0.962 | 0.942 | 0.938 |
| Level 2 | 0.0923 | 0.0950 | 0.0932 | 0.0924 |

Each average exposure level was maintained at 90% and over of the nominated concentration levels.

*14.2 Bioconcentration factor*
BCFs are shown in Table 2 below:

**Table 2 (BCFs)**

| | 2 weeks | 4 weeks | 6 weeks | 8 weeks |
|---|---|---|---|---|
| Level 1 | 29<br>40 | 9.3<br>6.8 | 24<br>13 | 28<br>11 |
| Level 2 | 39<br>22 | 27<br>40 | 55<br>47 | 33<br>62 |

The BCFs of the test substance ranged from 6.8 to 40 at level 1 and from 22 to 62 at level 2.

*14.3 Observation results of test fish*
No abnormality in behaviour and appearance of the test fish were observed.

*15 Factors possibly affecting accuracy*
No adverse effects on the reliability of the test we

**Nominal and Measured Concentrations:**

Details on both nominal and measured concentrations use in all evaluated studies in the CLH report were requested. This issue was also addressed in the responses to the public consultation.

Actual concentrations of test substance were measured in all acute and long-term tested evaluated in the proposal, so results can be expressed as both nominal and measured. The results tables in the CLH report are mainly expressed in terms of nominal concentrations. However, the 'discussion' sections for each study report the details on measured concentrations.

In the CLH report, only the long-term toxicity to fish study (Knight B (2003)) didn't give details on measured concentrations. However, this information was provided in the responses to the public consultation.

Therefore, all study data should have details on measured concentrations of test substance.