Section 7.1.2.2 Annex Point/TNsG Annex IIIA, XII.2.1	**Biodegradation in freshwater – water/sediment degradation study** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.1.3 **Annex Point/TNsG** **Annex IIIA, XII, 2.2**	**Adsorption/desorption screening test** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ]	**Technically not feasible** [ ] **Scientifically unjustified** [4]	
Limited exposure [4]	**Other justification** [ ]	
Detailed justification:	This data end point is not applicable to carbon dioxide as a biocide as used by Rentokil Initial. In water, carbon dioxide breaks down to give carbonic acid, which is brought about by the result of simple dissolution of the carbon dioxide in water. $CO_2 + H_2O \leftrightarrow H_2CO_3$ It will attain equilibrium with air spaces in soil through passive diffusion. The carbon dioxide used here is not applied directly to the sediment. The normal working practices of carbon dioxide as an insecticide fumigant are within a sealed enclosure (fumigation bubble) and therefore additional exposure to the gas is not expected.	
Undertaking of intended data submission [ ]	Not applicable	

[redacted]

Section 7.1.3 Annex Point/TNsG Annex IIIA, XII, 2.2	**Adsorption/desorption screening test** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FROM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.1.4 **Annex Point/TNsG** **Annex IIIA, XII. 2.2**	**Further studies on adsorption and desorption in water/sediment systems and, where relevant, on the adsorption and desorption of metabolites and degradation products where the preliminary risk assessment indicates that it is necessary** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ]	**Technically not feasible** [ ] **Scientifically unjustified** [✓]	
Limited exposure [✓]	**Other justification** [ ]	
Detailed justification:	This data end point is not applicable to carbon dioxide as a biocide as used by Rentokil Initial. In water, carbon dioxide breaks down to give carbonic acid, which is brought about by the result of simple dissolution of the carbon dioxide in water. $CO_2 + H_2O \leftrightarrow H_2CO_3$ It will attain equilibrium with air spaces in soil through passive diffusion. The carbon dioxide used here is not applied directly to the sediment. The normal working practices of carbon dioxide as an insecticide fumigant are within a sealed enclosure (fumigation bubble) and therefore additional exposure to the gas is not expected.	
Undertaking of intended data submission [ ]	Not applicable.	

[redacted]

Section 7.1.4 **Annex Point/TNsG** **Annex IIIA, XII. 2.2**	**Further studies on adsorption and desorption in water/sediment systems and, where relevant, on the adsorption and desorption of metabolites and degradation products where the preliminary risk assessment indicates that it is necessary** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FROM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.1.4.1 **Annex Point/TNsG** **Annex IIIA, XII. 2.1**	**Field study on accumulation in the sediment** Section 7: Ecotoxicological Profile including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	This data end point is not applicable to carbon dioxide as a biocide as used by Rentokil Initial. Carbon dioxide will attain equilibrium with air spaces in soil through passive diffusion. The carbon dioxide used here is not applied directly to the sediment. The normal working practices of carbon dioxide as an insecticide fumigant are within a sealed enclosure (fumigation bubble) and therefore additional exposure to the gas is not expected.	

[redacted]

Undertaking of intended data submission [ ]	Not applicable.	

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FROM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.1 **Annex Point/TNsG** **Annex IIIA, VII.4, XII.1.1**	**Aerobic degradation in soil, initial study** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ]	**Technically not feasible** [ ] **Scientifically unjustified** [✓]	
Limited exposure [✓]	**Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion. [redacted]	
Undertaking of intended data submission [ ]	Not applicable.	

Section 7.2.1 Annex Point/TNsG Annex IIIA, VII.4, XII.1.1	**Aerobic degradation in soil, initial study** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A7.2.1
Annex Point IIIA, VII.4, XII.1.1

Aerobic degradation in soil, initial study

Official use only

1. REFERENCE

Already submitted for carbon dioxide dossier for Product Type 14. Amendments made to the section on deficiencies.

1.1 Reference [redacted]

1.2 Data protection [redacted]

1.2.1 Data owner [redacted]

1.2.2

1.2.3 Criteria for data protection [redacted]

2. GUIDELINES AND QUALITY ASSURANCE

2.1 Guideline study
No.
Not carried out to OECD Guideline 106 and E.C test method C.18

2.2 GLP
No.
No information available whether study complies with the requirements of GLP.

2.3 Deviations
Yes.
No set guideline followed.

3. MATERIALS AND METHODS

3.1 Test material [redacted]

3.1.1. Lot/Batch number [redacted]

3.1.2 Specification [redacted]

[redacted]

3.2 Degradation products
Not reported.

3.2.1 Method of analysis for degradation products
Not applicable.

3.3 Reference substance
No.

3.3.1 Method of analysis for reference substance
Reference substance not used.

3.4 Soil types
See table A7_2_1-1 at end of this study summary for details of study area.

3.5	**Testing procedure**	
3.5.1	Test system	Field observation of soil carbon dioxide concentration began in May 1993 at the Oak site (See table A7_2_1-1 at end of this study summary for details), and May 1995 at the Larch site (see table A7_2_1-1 at end of this study summary for details). The reported submitted gives details of results obtained by September 1999. The concentration of carbon dioxide in soil air was measured at depths of 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0, 1.5, and 2.0 m below the ground surface.
3.5.2	Test solution and test conditions	Long term field observation of carbon dioxide in soil was conducted in a forested headwater basin. See table A7_2_1-1 at end of this study summary for details of study area.
3.6	**Test performance**	
3.6.1	Preliminary test	Not reported.
3.6.2	Screening test: Adsorption	Not reported.
3.6.3	Screening test: Desorption	Not reported.
3.6.4	HPLC method	Not reported.
3.6.5	Other test	Not reported.
		4 RESULTS
4.1	**Preliminary test**	
4.2	Screening test: Adsorption	Not reported.
4.3	Screening test: Desorption	Not reported.
4.4	Calculations	Based on results of repeated carbon dioxide measurement at the ground surface, a constant concentration value of 0.05% in volume is assumed. The soil carbon dioxide concentration generally increased with depth and was highest 1.0-1.5 m below the ground surface. Except for the area just below the ground surface carbon dioxide concentration in soil air was always >0.1%. At a depth of 2.0m , it usually exceeded 0.3%. The maximum concentration of 0.87% at the Oak site was measured at 1.0m on 27th August 1988. At the Larch site the maximum was 0.65% at 1.5 m on 3 September 1999. The carbon dioxide concentrations at the Oak site were generally higher than those at the Larch site.
4.4.1	Ka, Kd	Not reported.
4.4.2	Ka_{oc}, Kd_{oc}	Not reported.
4.5	Degradation Product(s)	Not included in the protocol.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and Methods

The concentration of carbon dioxide in soil air was measured at depths of 0.1, 0.2, 0.3, 0.4, 0.5, 0.7, 1.0, 1.5, and 2.0 m below the ground surface. The concentration of carbon dioxide in soil was determined with a Gastec gas detection device. A number of authors of previous studies have validated this method. At the beginning of the long-term observation, soil air collection probes were installed vertically to each depth with horizontal intervals of more than 0.3m. One probe was installed for each depth at each site. The probe consists of an 18 mm outer diameter PVC pipe with a 1.0 mm inner diameter tube inside, and is designed to enable the extraction of soil air from any depth without contamination or clogging. After pre-extraction of residual air from the collection probe, soil air was extracted from the probe and then introduced into the gas detection tube using a 100 ml hand pump. Carbon dioxide in the extracted air was immediately indicated by the length of the colour changed zone in the tube and recorded in the field. The concentration indicated by the gas detection tube was converted to % v/v carbon dioxide using a specific conversion calculation. Because the study area was located at elevations of more than 1500 m while the gas detection tube was calibrated for 1 atmosphere, atmospheric pressure correction was also needed.

Automatic data loggers at both sites continuously recorded atmospheric temperature and soil temperature at depths of 0.1, 0.3, 0.5, 0.7 and 1.0m. The pressure head of soil water was measured manually by tensiometers equipped with a mercury manometer at the depths of carbon dioxide measurements. Because the tensiometers froze, the pressure head could not be measured in winter. Daily precipitation was observed at Nobeyama, the Nagano Meterological Observatory Station nearest to the study area. Although a rain gauge is settled at the lower end of the experimental basin, it was inoperative from December to March. However, it has been confirmed that the amounts of rainfall observed at both rain gauges are similar during a snowless season.

Because the long-term field observation was conducted on a monthly basis, it is possible to show the typical seasonal patterns of carbon dioxide distribution in soil as a series of 12 carbon dioxide profiles, averaged monthly. A month was divided into three parts – the first 10 days, the middle 10 days and the last 8-10 days. The data was arithmetically averaged for each part of the month, and then a monthly average was calculated as the average of the three parts. In the absence of field observations for the first part of the month, the data obatined in the last part of the month was assigned. In the absence of field observations for the last part, the data from the first part of the next month was used. In the case of lack of data for the middle part of the month, the monthly mean was given by the average of the first and the last parts of the month. Several carbon dioxide profiles clearly different from the monthly trend were excluded from the calculation.

Using a similar procedure, monthly averaged data sets for soil temperature were compiled.

5.2	**Results and discussion**	
5.2.1	Adsorbed a.s [%]	Atmospheric temperatures observed at both sites were similar and mean annual temperature was 6.2°C for the observation period. The monthly mean temperature was highest in July or August (>15°C) and lowest in January or February (< -5°C). Annual temperature variations reached about 24°C, a characteristic feature of inland climate. The amount of precipitation is large in summer and small in winter. Soil temperatures at each depth were highest in August or September and lowest in February to April. Annual soil temperature variations ranged from 8-12°C at 1.0m deep to 16-18°C near the ground surface. Based on results of repeated carbon dioxide measurement at the ground surface, a constant concentration value of 0.05% in volume is assumed. The soil carbon dioxide concentration generally increased with depth and was highest 1.0-1.5 m below the ground surface. Except for the area just below the ground surface carbon dioxide concentration in soil air was always >0.1%. At a depth of 2.0m , it usually exceeded 0.3%. The maximum concentration of 0.87% at the Oak site was measured at 1.0m on 27th August 1988. At the Larch site the maximum was 0.65% at 1.5 m on 3 September 1999. The carbon dioxide concentrations at the Oak site were generally higher, than those at the Larch site.
5.2.2	K_a	Not reported.
5.2.3	K_d	Not reported.
5.2.4	Ka_{oc}	Not reported.
5.2.5	Ka/Kd	Not reported.
5.2.6	Degradation products (% of a.s)	Not included in the protocol.
5.3	**Conclusion**	The soil carbon dioxide concentration generally increased with depth and was highest 1.0-1.5 m below the ground surface. Except for the area just below the ground surface carbon dioxide concentration in soil air was always >0.1%. At a depth of 2.0m , it usually exceeded 0.3%.
5.3.1	Reliability	3
5.3.2	Deficiencies	Rather than looking at aerobic degradation of carbon dioxide in soil, this study measured normal, background levels of carbon dioxide in a typical soil.

Table A7_2_1-1: Description of study area

Location	Kawakami Experimental Basin. Mountainous headwater basin located at the east edge of Nagano Prefecture, central Japan.
Basin area	0.14 km^2 with elevations ranging from 1500 to 1690 m above sea level.
Atmospheric temperature	6.2°C (lower end of basin)
Annual precipitation	1450 mm (producing 830 mm of runoff)
Soil depth and description	Brown forest soil, 1.6m in depth. Neocene Meshimori-yama volcanic rocks underlie the soil mantle. A small ridge separates the basin into two smaller sub-basins, the North and South Valley.
Vegetation	Two types: a natural broadleaf forest composed mainly of oak (*Quercus mongolica* Fisch) and a Japanese larch (*Larix leptolepis* Gordon) plantation. Both forest floors are densely covered with bamboo grass (*Sasa nipponica)*. Two field observation sites, the Oak site and the Larch site were established to study soil carbon dioxide concentrations for both vegetation sites.
Description of observation site: Oak site	Sited in North valley, at an altitude of 1500m on the lower part of a steep southwest facing slope (average gradient 25°). Stand density is 9.4 trees per 100 m^2 average diameter at breast height (DBH) is 19.7 cm and tree height averages 13-16m. The average tree age is roughly 60 years. The soil at the Oak site has developed to a depth of nearly 6 m due to an accumulation of colluvium from the upper part of the slope. The A layer of the soil profile is 0.2-0.3 m thick, with the B layer following just under it, thin layers of angular gravel are found at several depths. The organic layer on the ground surface is not continuous.
Description of observation site: Larch site	Sited at the lower end of the basin, at an altitude of 1510 m on a relatively gentle north-facing slope (average gradient 15°). The Japanese Larch was planted in 1964-1965. The averages of stand density, diameter at breast height (DBH) and tree height are 8.1 trees per 100 m^2, 21.3 cm and about 19 m respectively. Litter fall from Japanese larches thickly covers the forest floor. The A layer of the soil profile is 0.2-0.5m thick. Clods of weathered material are found below 1.5m, but no gravel layer is found in the profile.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted.
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Materials and Methods	*State if applicants version is acceptable, or indicate relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*
Results and discussion	*Adopt applicant's version or include revised version. If necessary, discuss relevant deviations from applicant's view referring to the (sub)heading numbers.*
Conclusion	Other conclusions: *(adopt applicant's version or include revised version)*
Reliability	*Based on assessment of materials and methods include appropriate reliability indicator.*
Acceptability	acceptable / not acceptable *(give reasons if necessary e.g. if a study is considered acceptable despite a poor reliability indicator. Discuss the relevance of deficiencies and indicate if repeat if necessary).*
Remarks	
	COMMENTS FROM …..
Date	*Give date of comments submitted.*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion* *Discuss if deviating from view of rapporteur member state. .*
Results and discussion	*Discuss if deviating from view of rapporteur member state.*
Conclusion	*Discuss if deviating from view of rapporteur member state.*
Reliability	*Discuss if deviating from view of rapporteur member state.*
Acceptability	*Discuss if deviating from view of rapporteur member state.*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.2 **Annex Point/TNsG** **Annex IIIA, XII.1.1**	**Aerobic degradation in soil, further studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

[redacted]

Section 7.2.2 Annex Point/TNsG Annex IIIA, XII.1.1	**Aerobic degradation in soil, further studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.2.1 **Annex Point/TNsG Annex IIIA, VII.4, XII.1.1, XII.1.4**	**The rate and route of degradation including identification of the processes involved and identification of any metabolites and degradation products in at least three soil types under appropriate conditions** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

[redacted]

Section 7.2.2.1 **Annex Point/TNsG** **Annex IIIA, VII.4, XII.1.1, XII.1.4**	**The rate and route of degradation including identification of the processes involved and identification of any metabolites and degradation products in at least three soil types under appropriate conditions** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.2.2 **Annex Point/TNsG** **Annex IIIA, XII.1.1,** **Annex VI, para 85**	**Field soil dissipation and accumulation** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

Section 7.2.2.2 Annex Point/TNsG Annex IIIA, XII.1.1, Annex VI, para 85	**Field soil dissipation and accumulation** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.2.3 **Annex Point/TNsG** **Annex IIIA, XII.1.4**	**Extent and nature of bound residues** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

Section 7.2.2.3 Annex Point/TNsG Annex IIIA, XII.1.4	**Extent and nature of bound residues** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.2.4 **Annex Point/TNsG** **Annex IIIA, XII.1.1**	**Other soil degradation studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

[redacted]

Section 7.2.2.4 Annex Point/TNsG Annex IIIA, XII.1.1	**Other soil degradation studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.3 **Annex Point/TNsG** **Annex IIIA, XII.1.2-1.3**	**Adsorption and mobility in soil, further studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

[redacted]

Section 7.2.3 Annex Point/TNsG Annex IIIA, XII.1.2-1.3	**Adsorption and mobility in soil, further studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.3.1 **Annex Point/TNsG** **Annex IIIA, XII.1.2**	**Adsorption and desorption in accordance with the new test guideline EC C18 or the corresponding OECD 106 and, where relevant, adsorption and desorption metabolites and degradation products** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

Section 7.2.3.1 **Annex Point/TNsG** **Annex IIIA, XII.1.2**	**Adsorption and desorption in accordance with the new test guideline EC C18 or the corresponding OECD 106 and, where relevant, adsorption and desorption metabolites and degradation products** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.2.3.2 **Annex Point/TNsG** **Annex IIIA, XII.1.3**	**Mobility in at least three soil types and where relevant mobility of metabolites and degradation products** Section 7: Ecotoxicological Profile, including Fate and Behaviour Already submitted for carbon dioxide dossier for Product Type 14.	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [✓] **Other justification** [ ]	
Detailed justification:	Carbon dioxide, as a biocide, as used by Rentokil Initial is not applied or emitted directly to the soil and therefore this study is not required. Data requirements A7.1.1.2.1 and A7.1.1.2.2 do not indicate the need to conduct studies on the fate and behaviour of carbon dioxide in soil and in addition, this is substantiated by the fact that carbon dioxide does undergo a degree of abiotic degradation by means of simple dissolution in water. Also, it is well known that although carbon dioxide occurs predominantly in air, it will attain equilibrium with air spaces in soil through passive diffusion.	
Undertaking of intended data submission [ ]	Not applicable.	

Section 7.2.3.2 Annex Point/TNsG Annex IIIA, XII.1.3	**Mobility in at least three soil types and where relevant mobility of metabolites and degradation products** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.3.1 **Annex Point/TNsG** **Annex IIIA, VII.5**	**Phototransformation in air (estimation method), including identification of breakdown products** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [ ]	**Technically not feasible** [ ] **Scientifically unjustified** [ ] **Other justification** [✓]	
Detailed justification:	As a gas, under all environmental conditions that are likely to occur on earth, carbon dioxide will occur predominantly in air. Carbon dioxide occurs as a by-product of aerobic respiration. There is a natural "Carbon Cycle" whereby carbon dioxide is continuously added and removed from the environment though natural processes. The report submitted under the data end point "7.1.1.1. Hydrolysis as a function of pH and identification of breakdown products" characterises the role, fate and behaviour of carbon dioxide in the environment.	
Undertaking of intended data submission [ ]	Not applicable.	

Section 7.3.1 Annex Point/TNsG Annex IIIA, VII.5	**Phototransformation in air (estimation method), including identification of breakdown products** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.3.2 **Annex Point/TNsG** **Annex IIIA, XII.3**	**Fate and behaviour in air, further studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ] **Limited exposure** [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [ ] **Other justification** [ ]	
Detailed justification:	As a gas, under all environmental conditions that are likely to occur on earth, carbon dioxide will occur predominantly in air. Carbon dioxide occurs as a by-product of aerobic respiration. There is a natural "Carbon Cycle" whereby carbon dioxide is continuously added and removed from the environment though natural processes. The report submitted under the data end point "7.1.1.1. Hydrolysis as a function of pH and identification of breakdown products" characterises the role, fate and behaviour of carbon dioxide in the environment.	

Undertaking of intended data submission [ ]	Not applicable.	

Section 7.3.2 Annex Point/TNsG Annex IIIA, XII.3	**Fate and behaviour in air, further studies** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FORM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section A7.4.1.1 **Annex Point/TNsG** **Annex IIA, VII 7.1**	**Acute toxicity to fish** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [ ]	
Limited exposure [ ]	**Other justification** [ ]	
Detailed justification:	An acute toxicity study for carbon dioxide to fish is not considered necessary for the following reason: Study summaries are available for two pieces of work investigating the effects of carbon dioxide to fish. One of which shows that dissolved concentrations of up to 6.3% carbon dioxide have not given rise to irreversible physiological and behavioural effects. *Refer to study summaries for details about the data available on the inhalation toxicity of carbon dioxide.* No key study available for this data end point that has a reliability indicator of 1 or 2. Studies are available which have a reliability indicator of 3. These data can be useful for the risk assessment, so have been included here. A rudimentary $PNEC_{water}$ value of 0.03% has been determined for carbon dioxide (i.e. normal atmospheric concentrations of carbon dioxide. [redacted]	
Undertaking of intended data submission [ ]	Not applicable	

Section A7.4.1.1 Annex Point/TNsG Annex IIA, VII 7.1	**Acute toxicity to fish** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FROM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A7.4.1.1 Acute toxicity to fish (1 of 2)

Annex Point IIA7.1

Official use only

1 REFERENCE

Already submitted for carbon dioxide dossier for Product Type 14. Amendments made to the section on deficiencies.

1.1	**Reference**	[redacted]
1.2	**Data protection**	[redacted]
1.2.1	**Data owner**	[redacted]
1.2.3	**Criteria for data protection**	[redacted]

2 GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	No. Not carried out to Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075.
2.2	**GLP**	No. No details available regarding the testing guidelines used.
2.3	**Deviations**	Yes. This study did not follow the test guidelines of Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075. Rather than looking at acute toxicity *per se,* this investigates the physiological and behavioural effects of fish exposed to carbon dioxide.

3 MATERIALS AND METHODS

3.1	**Test material**	[redacted]
3.1.1	Lot/Batch number	[redacted]
3.1.2	Specification	[redacted]

Section A7.4.1.1 Acute toxicity to fish (1 of 2)

Annex Point IIA7.1

3.1.3	Purity	[redacted]
3.1.4	Composition of product	Not applicable for active substance.
3.1.5	Further relevant properties	Not reported.
3.1.6	Method of analysis	Not reported.
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not applicable.
3.3	**Reference substance**	No.
3.3.1	Method of analysis for reference substance	Not applicable as reference substance was not used.
3.4	**Testing procedure**	

3.4.1 Dilution water

Criteria	Details
Source	Not reported.
Alkalinity	23.5 mg/L
Hardness	35.1 mg/L
pH	6.8
Oxygen content	11.2 mg/L
Conductance	89 µmhos
Holding water different from dilution water	Laboratory holding and test waters have same properties.

Section A7.4.1.1 Acute toxicity to fish (1 of 2)

Annex Point IIA7.1

3.4.2 Test organisms

Criteria	Details		
Species / strain	Brook trout *Salvelinus fontinalis*	Slimy Sculpin *Cottus cognatus*	Blacknose dace *Rhinichthys atratulus*
Source	Private hatchery	Local second -order streams	Local second - order streams
Wild caught	No	Yes – no other details given	Yes – no other details given
Age / size	Age not given 170 ± 11 mm total length	Age not given 70 ± 4 mm total length	Age not given 69 ± 4 mm total length
Kind of food	Dry salmon diet (ASD2-30)	Locally caught (live cut) earthworms	Dry salmon diet (ASD2-30), live earth worms and brine shrimp nauplii
Amount of food	1g per tank in total.	12-14 cm long earthworms were cut in half and distributed uniformly around the tank.	Would not feed.
Feeding frequency	Not reported.	Not reported.	Would not feed.
Pretreatment	7-10 days acclimation period.	7-10 days acclimation period.	7-10 days acclimation period.
Feeding of animals during test	Fed 1 pellet of ASD2-30 at a time. Up to a total of 1 g per tank.	Not reported.	Would not feed.

3.4.3 Test system

Criteria	Details
Test type	Flow through.
Renewal of test solution	Flow through maintained at 6 L / min.
Volume of test vessels	Volume of test vessels not reported, but water volume maintained at 85 L.
Volume/animal	Not reported.
Number of animals/vessel	20 Blacknose dace / vessel 10 Slimy sculpin / vessel 10 Brook trout / vessel
Number of vessels/concentration	4 treatment levels of carbon dioxide with 3 replicates of each, giving 12 in total. Control tank: 0.0 ±0.0% dissolved CO_2 Low: 1.4 ± 0.4% dissolved CO_2 Medium: 2.8 ± 0.6% dissolved CO_2 High: 5.1 ± 1.3% dissolved CO_2

	Test performed in closed vessels due to significant volatility of TS	Not reported.	

Section A7.4.1.1 Acute toxicity to fish (1 of 2)

Annex Point IIA7.1

3.4.4	Test conditions	**Criteria**	**Details**
		Test temperature	9°C – measurements conducted during test not actually reported, but largely unaffected.
		Dissolved oxygen	11.2 mg/L - measurements conducted during test not actually reported, but largely unaffected.
		pH	6.8 – during treatment, pH decreased to 5.5 except in the control vessels.
		Adjustment of pH	Not reported.
		Aeration of dilution water	Not reported.
		Intensity of irradiation	Not reported.
		Photoperiod	Not reported.
3.4.5	Duration of test	24 hours.	
3.4.6	Test parameter	Blood physiological variables (including hematocrit levels), behaviour including feeding responses before, during and after exposure.	
3.4.7	Sampling	Dissolved CO_2 concentrations measured throughout the 24 hour treatment period using a model GD-444 CEA instrument gas monitor with gas sampling pump.	
3.4.8	Monitoring of TS concentration	CO_2 levels monitored periodically during the 24-hour treatment period.	
3.4.9	Statistics	One-way analysis of variance (ANOVA) carried out on the behavioural activities.	

4 RESULTS

4.1	**Limit test**	Not performed.
4.1.1	Concentration	Not required.
4.1.2	Number / percentage of animals showing adverse effects	Not required.
4.1.3	Nature of adverse effect	Not required.
4.2	**Results test substance**	
4.2.1	Initial concentration of substance	Not reported.
4.2.2	Actual concentration of test substance	The experiment was designed to cover the exposure to carbon dioxide at three levels: 1.4 ± 0.4% (low), 2.8 ± 0.6% (moderate) and 5.1 ± 1.3% (high).
4.2.3	Effect data (mortality)	Mortality was not being investigated in this study.
4.2.4	Concentration / response curve	Not relevant.
4.2.5	Other effects	Effects were observed on hematocrit levels, plasma glucose levels, ventilation rates, pectoral fin beats and cough rates.
4.3	**Results of controls**	
4.3.1	Number / percentage of animals showing adverse effects	The number / percentage of animals showing adverse effects was not directly reported. However, the proportional (and percentage) of behavioural variables that showed post-treatment recovery to pre-treatment rates after exposure to various treatment levels of dissolved CO_2 is shown below:

Treatment level	Brook trout	Slimy sculpin	Blacknose dace	All species
Control		0/1 (0%)	1/1 (100%)	1/2 (50%)
Low	0/1 (0%)			0/1 (0%)
Medium	1/2 (50%)	1/2 (50%)	5/6 (83%)	7/10 (70%)
High	3/7 (43%)	4/5 (8%)	5/5 (100%)	12/17 (71%)
All levels	4/10 (40%)	5/8 (50%)	11/12 (92%)	20/30 (67%)

4.3.2	Nature of adverse effects	This study was not specifically looking for adverse effects. It was looking at behavioural effects such as ventilation rates, pectoral fin beats and cough rates. Other effects on hematocrit levels and plasma glucose were seen.
4.4	**Test with reference substance**	
4.4.1	Concentration	Test not performed.

4.4.2	Results	Test not performed.

Section A7.4.1.1 Acute toxicity to fish (1 of 2)
Annex Point IIA7.1

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

This study was not carried out to Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075.

Rather than looking at acute toxicity *per se*, this investigates the physiological and behavioural effects of fish exposed to carbon dioxide.

Brook trout, blacknose dace and slimy sculpin were tested in a system designed to stimulate an acute exposure to CO_2. Three replicates of four different CO_2 levels were tested in treatment vessels. Substrate cover of flat creekbed stones (5-15 cm) was provided in each tank; flow maintained at 6 L/minute and water volume at 85 L. Blacknose dace and slimy sculpin were collected from local second-order streams and brook trout from a private hatchery. After 2 days in laboratory holding tank, fish were anaesthetised, measured, sorted and distributed into tanks to give 20 blacknose dace, 10 slimy sculpin and 10 brook trout per tank. Numbers and weight of fish were designed to provide adequate samples of blood and behaviour without overcrowding.

General water quality from source to experimental tanks was similar and all fish were allowed 7 – 10 days acclimation to test tanks during which they were observed for signs of parasites and other pathology. All fish were offered suitable diets, although blacknose dace would not feed throughout.

Dissolved CO_2 was administered to 4 reservoir tanks from gas cylinders. In each reservoir tanks, 9°C well water was mixed with the CO_2 treated water to obtain test levels. The reservoir tanks then supplied each replicate tank.

Percentage CO_2 readings, pH and other water quality variables were measured.

All 3 fish species were exposed to treatment levels of control ($0.0 \pm 0.0\%$), low ($1.4 \pm 0.4\%$), medium ($2.8 \pm 0.6\%$), and high ($5.1 \pm 1.3\%$) dissolved CO_2 concentrations for 24 hours. CO_2 concentration was measured throughout the 24-hour period, and adjustments made periodically to maintain treatments at or near prescribed points. After tests, fish were monitored for a 1-week period to assess short-term mortality.

5.2 Results and discussion

Physiological responses differed by species.
All species had elevated hematocrits after 1 hour of exposure.
Brook trout plasma glucose levels were raised after 1 hour.
All species showed increased branchial ventilation, indicating stress although acclimation was indicated in blacknose dace after 24 hours.
Brook trout had the longest reaction to stress at lower carbon dioxide levels.

Recovery to pre-treatment activity rates of most behaviour patterns (including feeding) was observed 24 hours after cessation of exposure in

		all three species.

Section A7.4.1.1 Acute toxicity to fish (1 of 2)

Annex Point IIA7.1

5.2.1	LC_0	Not reported.
5.2.2	LC_{50}	Not reported.
5.2.3	LC_{100}	Not reported.
5.3	**Conclusion**	Rather than looking at acute toxicity *per se,* this test investigated the physiological and behavioural effects of 3 species of fish exposed to carbon dioxide. The results show that physiological responses to increased carbon dioxide in fish differed by species when they were exposed to 1.4%, 2.8% and 5.1% carbon dioxide. However, recovery to pre-treatment activity rates of most behaviour patterns (including feeding) was observed 24h after cessation of exposure in all 3 test species.
5.3.1	Other conclusions	None made.
5.3.2	Reliability	3
5.3.2	Deficiencies	Yes. This study was not carried out to Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075. Rather than looking at acute toxicity *per se,* this investigates the physiological and behavioural effects of fish exposed to carbon dioxide.

Section A7.4.1.1 Acute toxicity to fish (1 of 2)

Annex Point IIA7.1

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Materials and methods	*Adopt applicant's version or include revised version. If necessary, discuss relevant discrepancies referring to the (sub) heading numbers and to applicant's summary and conclusion.*
Results and discussion	*Adopt applicant's version or include revised version, If necessary, discuss relevant deviations from applicant's view referring to the (sub)heading numbers*
Conclusion	*Adopt applicant's version or include revised version*
Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator (the text in section 4.4.2.5.1 gives guidance on this point)*
Acceptability	Acceptable / not acceptable *(give reasons if necessary, e.g. if a study is considered acceptable despite a poor reliable indicator. Discuss the relevance of deficiencies and indicate if repeat is necessary.)*
Remarks	
	COMMENTS FROM
Date	*Give date of the comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*

Section A7.4.1.1 Acute toxicity to fish (2 of 2)

Annex Point IIA7.1

Official use only

1 REFERENCE

Already submitted for carbon dioxide dossier for Product Type 14. Amendments made to the section on deficiencies.

1.1 Reference [redacted]

1.2 Data protection [redacted]

1.2.1 Data owner [redacted]

1.2.3 Criteria for data protection [redacted]

2 GUIDELINES AND QUALITY ASSURANCE

2.1 Guideline study

No

Not carried out to Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075.

2.2 GLP

No

GLP was not compulsory at the time the study was performed.

2.3 Deviations

Yes.

This study did not follow the test guidelines of Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075. Rather than looking at acute toxicity *per se*, this report investigates the survival of the fish species at various concentrations of carbon dioxide.

3 MATERIALS AND METHODS

3.1 Test material [redacted]

3.1.1 Lot/Batch number [redacted]

3.1.2 Specification [redacted]

3.1.3 Purity [redacted]

Section A7.4.1.1 Acute toxicity to fish (2 of 2)

Annex Point IIA7.1

3.14	Composition of product	Not applicable for active substance.
3.15	Further relevant properties	Not reported.
3.16	Method of analysis	Not reported.
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not applicable. Carbon dioxide obtained and used from commercially available cylinder.
3.3	**Reference substance**	No.
3.3.1	Method of analysis for reference substance	Not applicable as reference substance was not used.
3.4	**Testing procedure**	

3.4.1 Dilution water

Criteria	**Details**
Source	Tap water.
Alkalinity	Not reported.
Hardness	Not reported.
pH	Not reported.
Oxygen content	Experiments with trout designed to cover range of concentrations of dissolved oxygen from 0.5 to 10 ppm.
Conductance	Not reported.
Holding water different from dilution water	Not reported.

3.4.2 Test organisms

Criteria	**Details**	
Species / strain	Rainbow trout *Salmo gairdnerii* Richardson.	Perch *Perca fluviatilis* L.
Source	Commercial trout farm.	Channels supplying rapid sand filters of the Hampton water works of the Metropolitan water board.
Wild caught	No.	Yes. Channels supplying rapid sand filters of the Hampton water works of the Metropolitan Water Board.
Age / size	About 6 months. 6.9 ± 0.7 cm.	No age reported. 11.9 ± 2.2 cm.
Kind of food	No food given.	No food given.
Amount of food	No food given.	No food given.
Feeding frequency	No food given.	No food given.
Pretreatment	Acclimatised to test temperatures for 24 hours prior to test.	Acclimatised to test temperatures for 24 hours prior to test.

	Feeding of animals during test	No	No.	

Section A7.4.1.1 Acute toxicity to fish (2 of 2)

Annex Point IIA7.1

3.4.3	Test system	**Criteria**	**Details**
		Test type	Not reported.
		Renewal of test solution	Not reported.
		Volume of test vessels	40 litres.
		Volume/animal	Not reported.
		Number of animals/vessel	10 trout or 5 trout and 5 perch per vessel.
		Number of vessels/concentration	3 vessels at 12.5°C, 16.5°C and 19.5°C. Same quantity of gas in each: 0.5 – 10 ppm dissolved oxygen and 0 – 240 ppm carbon dioxide.
		Test performed in closed vessels due to significant volatility of TS	Yes. Vessels sealed form atmosphere by a glass plate positioned on top.
3.4.4	Test conditions	**Criteria**	**Details**
		Test temperature	12.5°C, 16.5°C and 19.5°C ± 0.5°C.
		Dissolved oxygen	0.5 – 10 ppm ± 5°C.
		pH	Not reported.
		Adjustment of pH	Not reported.
		Aeration of dilution water	Not reported.
		Intensity of irradiation	Not reported.
		Photoperiod	Not reported.
3.4.5	Duration of test	Up to 24 hours (if any fish remained alive).	
3.4.6	Test parameter	Mortality.	
3.4.7	Sampling	Not reported.	
3.4.8	Monitoring of TS concentration	Carbon dioxide concentration estimated by determining the pH value, temperature and bicarbonate alkalinity of a sample and calculating the free carbon dioxide concentration using predetermined nomograms.	
3.4.9	Statistics	Standard deviations and means of the logarithms of periods of survival were assessed.	
		4 RESULTS	
4.1	**Limit test**		
4.1.1	Concentration	Test not performed.	
4.1.2	Number / percentage of animals showing adverse effects	Test not performed.	

4.1.3	Nature of adverse effect	Test not performed.

Section A7.4.1.1 Acute toxicity to fish (2 of 2)

Annex Point IIA7.1

4.2	**Results test substance**	
4.2.1	Initial concentration of substance	Not reported, however the experiment was designed to cover a range of concentrations of carbon dioxide from 0 – 240 ppm.
4.2.2	Actual concentration of test substance	Not reported, however the experiment was designed to cover a range of concentrations of carbon dioxide from 0 – 240 ppm.
4.2.3	Effect data (mortality)	All fish died within 24 hours, ie 100% mortality.
4.2.4	Concentration / response curve	Not reported.
4.2.5	Other effects	Not reported.
4.3	**Results of controls**	
4.3.1	Number / percentage of animals showing adverse effects	Not reported.
4.3.2	Nature of adverse effects	Not reported.
4.4	**Test with reference substance**	
4.4.1	Concentration	Test not performed.
4.4.2	Results	Test not performed.

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and methods

This study was not carried out to Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075. Rather than looking at acute toxicity *per se*, this report investigates the survival of the fish species at various concentrations of carbon dioxide.

The experiments were designed to cover a range of concentrations of 0 - 240 ppm carbon dioxide at temperatures of 12.5°C, 16.5°C and 19.5°C.

The desired concentrations of oxygen and carbon dioxide were obtained by passing through the test aquarium mixtures of nitrogen, oxygen and carbon dioxide in suitable proportions at a rate of about 50ml/second (monitored by a flow meter). This rate ensured that there were no appreciable alterations in the concentrations of oxygen and carbon dioxide due to the respiration of the fish. The three aquarium were filled with tap water and the tops sealed from the atmosphere with glass plates so that the surface of the water was in contact with the gas mixture (the excess of which was led to waste through a small exhaust vent). The temperature of the aquariums was maintained within ± 0.5°C.

Concentrations of dissolved oxygen and carbon dioxide were monitored throughout the tests. Fish, in groups of 10 were tested in each aquarium (10 trout or 5 trout and 5 perch). They were sorted into batches at random and acclimatised at the appropriate temperature without food for 24 hours

		prior to testing. The period of survival for each fish was recorded as minutes between immersion of the fish in a test aquarium and the time at which all movements, respiratory and otherwise, had ceased.

5.2	**Results and discussion**	It is shown that concentrations of carbon dioxide that sometimes occur in polluted streams can more than double the minimum concentration of dissolved oxygen necessary for the survival of half a population of rainbow trout fingerlings for 24 hours. Increase in temperature between 12.5°C and 19.5°C shortens period of survival in solutions containing up to 67 ppm carbon dioxide.
5.2.1	LC_0	Not reported.
5.2.2	LC_{50}	Not reported.
5.2.3	LC_{100}	Not reported.
5.3	**Conclusion**	Not reported.
5.3.1	Other conclusions	Not reported
5.3.2	Reliability	3
5.3.2	Deficiencies	Yes This study was not carried out to Guideline C.1 in Annex V of Directive 67/548/EEC, to OECD Guideline 203, or to US-EPA guideline OPPTS 850.1075. Rather than looking at acute toxicity *per se*, this report investigates the survival of the fish species at various concentrations of carbon dioxide.

Section A7.4.1.1 Acute toxicity to fish (2 of 2)

Annex Point IIA7.1

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Materials and methods	*Adopt applicant's version or include revised version. If necessary, discuss relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*
Results and discussion	*Adopt applicant's version or include revised version, If necessary, discuss relevant deviations from applicant's view referring to the (sub)heading numbers*
Conclusion	*Adopt applicant's version or include revised version*
Reliability	*Based on the assessment of materials and methods include appropriate reliability indicator (the text in section 4.4.2.5.1 gives guidance on this point)*
Acceptability	Acceptable / not acceptable *(give reasons if necessary, e.g. if a study is considered acceptable despite a poor reliable indicator. Discuss the relevance of deficiencies and indicate if repeat is necessary.)*
Remarks	
	COMMENTS FROM
Date	*Give date of the comments submitted*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.* *Discuss if deviating from view of rapporteur member state*
Results and discussion	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Reliability	*Discuss if deviating from view of rapporteur member state*
Acceptability	*Discuss if deviating from view of rapporteur member state*

Table 4-2: Standard form for justification of the non-submission of data

Section A7.4.1.2 **Annex Point/TNsG** **Annex IIA, VII 7.2**	**Acute toxicity to invertebrates** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only

Other existing data	[✓]	**Technically not feasible**	[ ]	**Scientifically unjustified**	[ ]
Limited exposure	[ ]	**Other justification**	[ ]		

Detailed justification:

An acute toxicity study for carbon dioxide to invertebrates is not considered necessary for the following reason:

There are two studies available in the public domain which investigates the effects of carbon dioxide to *Daphnia magna* and aquatic cladocera species, including *Daphnia similis*. This study shows that *Daphnia similis* could tolerate carbon dioxide levels of 86 mg/L in water (86 ppm, or 0.0086%). *Monia flagellata* and *Ceriodaphnia rigaudi* could tolerate 12.0 mg/L (12 ppm or 0.0012 %) and 32.6 mg/L (32.6 ppm or 0.0326 mg/L). *Daphnia magna* can tolerate levels of carbon dioxide up to 2% v/v. The carbon dioxide values cited here are naturally occurring exposures.

Refer to attached study summary for details about the data available on the toxicity of carbon dioxide to aquatic invertebrates.

In addition, a study is available in the public domain that determined the levels of carbon dioxide found in nests of the mud-dwelling mangrove ant, *Polyrhachis sokolova*. Carbon dioxide concentrations in the nests of the mangrove ant are approximately 2%, but can reach 11% during and immediately after the nest is covered in water at high tide. While this data does not consider toxicity to aquatic species (nor are the figures from this study used in the risk assessment) it provides some useful supporting information about the toxicity of carbon dioxide to invertebrate species.

No key study available for this data end point that has a reliability indicator of 1 or 2. Studies are available which have a reliability indicator of 3. These data can be useful for the risk assessment, so have been included here. rudimentary $PNEC_{water}$ value of 0.03% has been determined for carbon dioxide (i.e. normal atmospheric concentrations of carbon dioxide.

Section A7.4.1.2 **Annex Point/TNsG** **Annex IIA, VII 7.2**	**Acute toxicity to invertebrates** Section 7: Ecotoxicological Profile, including Fate and Behaviour

Undertaking of intended data submission	[ ]	Not applicable

Evaluation by Competent Authorities
Use separate "evaluation boxes" to provide transparency as to the Comments and views submitted
EVALUATION BY RAPPORTEUR MEMBER STATE

Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FROM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Section A7.4.1.2
Annex Point IIA, VII, 7.2

Acute toxicity to invertebrates (1 of 3)

Special investigation in *Daphnia magna*

Official use only

		1. REFERENCE
		Already submitted for carbon dioxide dossier for Product Type 14. Amendments made to the section on deficiencies.
1.1	**Reference**	[redacted]
1.2	**Data protection**	[redacted]
1.2.1	Data owner	[redacted]
1.2.2		
1.2.3	Criteria for data protection	[redacted]
		2. GUIDELINES AND QUALITY ASSURANCE
2.1	**Guideline study**	No. Not carried out to EC Method C2 in Annex V of Directive 67/548/EEC.
2.2	**GLP**	No. No information available on whether study complies with the requirements of GLP.
2.3	**Deviations**	Yes. No set guideline followed.
		3. MATERIALS AND METHODS
3.1	**Test material**	[redacted]
3.1.1.	Lot/Batch number	[redacted]
3.1.2	Specification	[redacted]
		[redacted]
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not applicable. No special procedures were applied to determine carbon dioxide content in water.
3.3	**Reference substance**	No.
3.3.1	Method of analysis for reference substance	Reference substance not used.
3.4	**Testing procedure**	
3.4.1	Dilution water	1:14 diluted seawater. No other information reported.
3.4.2	Test organisms	Female *Daphnia magna* Straus Refer to table A7_1_1_2-1 for description of test organisms.
3.4.3	Test system	Refer to table A7_1_1_2-2 for description of test system.

Section A7.4.1.2 **Acute toxicity to invertebrates (1 of 3)**
Annex Point IIA, VII, 7.2 Special investigation in *Daphnia magna*

3.4.4	Test conditions	Refer to table A7_1_1_2-3 for description of test conditions.	
3.4.5	Duration of test	30 minutes.	
3.4.6	Test parameter	Heart rate, and pH changes.	
3.4.7	Sampling	Refer to table A7_1_1_2-3 for description of test conditions.	
3.4.8	Monitoring of TS concentration	Refer to table A7_1_1_2-3 for description of test conditions.	
3.4.9	Statistics	Not reported.	
		4 RESULTS	
4.1	**Limit Test**	Not performed.	
4.2	**Results test substance**		
4.2.1	Initial concentrations of test substance	Carbon dioxide concentrations were increased to 2% (oxygen concentration were either at normal levels, or decreased to 3.6%). Note that author comments that *Daphnia* are known to be naturally exposed to ambient carbon dioxide concentrations of up to 2%.	祺 4.2.2
4.2.2	Actual concentrations of test substance	Carbon dioxide concentrations were increased to 2% (oxygen concentration were either at normal levels, or decreased to 3.6%). Note that author comments that *Daphnia* are known to be naturally exposed to ambient carbon dioxide concentrations of up to 2%.	
4.2.3	Effect data (Immobilisation)	Heart rate changes could not be provoked by P_{CO2} changes during normoxia (normal levels of oxygen). However, during severe hypoxia (low oxygen concentration of 3.6%), the application of hypercapnia (2% carbon dioxide, pH 6) caused a decrease of heart rate of *Daphnia magna* by 20-50 beats per minute. The minimum was reached within a few minutes. After 20 minutes, the heart had returned back to a rate similar to the pre-hypercapnic value (the normocapnic value at pH 7.45). Switching then to normocapnia or acapnia (pCO2: 0%, pH 8.5) caused the heart rate either to non-transiently or transiently to increase. Concerning blood pH, the application of hypercapnia (2% carbon dioxide, pH 6) caused a transient decrease of pH even during normoxia. The minimum was reached within 10 minutes. After 20-30 minutes the pH had increased to a stable value which was a little lower than the pre-hypercapnic one.	
4.2.4	Concentration/ response curve	Not reported.	
4.2.5	Other effects	None reported.	
4.3	**Results of controls**	No control species reported.	
4.4	**Test with reference substance**	Not performed.	
		5 APPLICANT'S SUMMARY AND CONCLUSION	
5.1	**Materials and Methods**	This study was not carried out to EC Method C2 in Annex V of Directive 67/548/EEC.	

(Continued....)

Section A7.4.1.2 **Acute toxicity to invertebrates (1 of 3)**
Annex Point IIA, VII, 7.2 Special investigation in *Daphnia magna*

5.1 Materials and Methods

(Continued)

Female individuals of *Daphnia magna* Straus were used. In most experiments, the animals had a body length of 2.5 +/- 0.2 mm (measured from the anterior most part of the head to the base of the apical spine), and a body mass of approximately 1 mg. The animals descended from a clone (clone 5) cultured at the University of Sheffield, Department of Animal and Plant Physiology, United Kingdom.

An animal chamber made of anodized aluminium was utilised for the experiments. Two cover slides were used as top and bottom of the inner room, whose lateral wall was the inside of the water filled theromstated casing. A constant medium flow (8-10 ml min^{-1}) through the chamber was generated by a peristaltic pump located after it. The test solution (1:14 diluted seawater) was sucked up from a thermostated glass vessel with a small opening (transport time from vessel to chamber 6s), where it was equilibrated with different O_2/N_2 mixtures (at normocapnic conditions 400 ppm CO_2), using gas mixing pumps. Temperature and oxygen content of the medium leaving the chamber was continually checked using a needle-shaped thermoelement (NiCr-Ni Sensortek, Clifton NJ) and a small oxygen electrode (P/N SI 130, Strathkelvin Instruments Glasgow, UK).

Single *Daphnia magna* were fixed in the chamber by gently screwing the chamber's top (which has a fine thread) down. The animals were acclimated in the chamber for about 1h. Within the first 30 minutes of acclimation the heart rate usually reached a constant level. At the beginning of the actual experiment the animals were further exposed to normoxia (normal levels of oxygen) / normocapnia (normal levels of carbon dioxide) for 15-20 minutes. Then, after switching the gas-mixing pump, the medium in the thermostated glass vessel was equilibrated with nitrogen (at normal levels of carbon dioxide) to remove oxygen (Anoxia: oxygen levels < 0.06 %). Anoxia was not instantaneously reached in the medium perfusing the animal chamber, and a short hypoxic period (where there was a deficiency in the amount of oxygen reaching body tissues) occurred for approximately 10 minutes. After the experiments on the physiological responses to short or long-term anoxia (absence of oxygen), normal levels of oxygen (normoxia) was applied again.

Heart rate was evaluated by video microscopy and digital image processing, utilising the rhythmic variations in pixel intensity. The amplitude of heart wall movements in fixed animals, being a relative measure of stroke volume, was evaluated utilising video recordings. During the experiments on circulation, the medium's temperature was 15°C.

The transparency of *Daphnia magna* allowed the application of pH sensitive dyes to determine pH changes. The diluted dyes were injected into the blood capillaries (1B100F-4, WP1 Sarasota FL) were pulled (micropipette puller from Zaschka Zoological Institute of the University of Munich Germany) and bevelled (beveller 1300M, WPI Sarasota) to an angle of 30° between capillary and a

(Continued....)

Section A7.4.1.2 **Acute toxicity to invertebrates (1 of 3)**
Annex Point IIA, VII, 7.2 Special investigation in *Daphnia magna*

5.2 Materials and Methods

(Continued)

0.3 µm aluminium oxide coated film (3 M Neuss, Germany), resulting in a diameter of the opening of 9 –10 µm. The pointed shape made penetration of the cuticle easier. Micropipette movements were controlled via a motor driven micromanipulator (AM3 DC-K, control unit BA-ST 3, Bachofer Reutlingen Germany). Injection pressure was provided by a microinjector (Transjector 5246, Eppendorf Hamburg, Germany). During injection, the animals (being in a small droplet of medium) became shortly immobilised, because the micropipette pressed them lightly to a semi-circular silicone support on a microscope slide. Another technique was to briefly fix the apical spine to the slide using wax. For that, the medium around the spine was shortly removed. Approximately 10 ml of dye solution (which is approximately 1.5% of total blood space) was injected into a dorsal lacuna carrying blood directly to the heart. Direct observation of the heart showed that such small, injected volumes did not cause any significant heart rate changes. After the experiments, the animals showed normal swimming patterns. In control experiments, the animals tolerated injection volumes ten times higher.

Blood pH changes were measured at temperatures of 20°C using the pH sensitive fluorescent dye BCECF (Molecular Probes Eugene OR). A stock solution of BCECF in dimethyl sulphoxide was stored at -20°C. Before injecting, the stock solution was diluted with Ringer's solution to a final concentration of 1 mM BCECF. Monochromatic light was generated using a rapid scanning monochromator equipped with xenon arc lamp (TILL Phototonics, Planegg Germany). The fluorescence image (of the anterior body part of *Daphnia*) was detected with a liquid-nitrogen cooled CCD camera which took 2 frames per second (LN/CCD-576E Princeton Instruments Trenton NJ). As dye concentration in different body parts may vary, the measurements were done in excitation ratio mode (excitation 439 nm and 490 nm, emission measured above 520 nm). Being interested in pH changes and not absolute values, time-consuming calibration procedures were not applied.

Confocal laser microscopy was used to image the extra and intracellular distribution of pH sensitive dyes in *Daphnia*, and pH changes during anoxia (oxygen deficiency), was studied (at 20°C). Two different kinds of the pH sensitive fluorescent dye SNARF-1 (molecular probes) were used for the emission ratio measurements, (excitation at 488 nm, emissions measured at 588 and 604 nm):
(i) to image blood spaces only and to prevent the penetration of dye into cells, the dextran-coupled indicator (dextran, SNARF-1, 70,000 MW anionic), was applied.
(ii) for an imaging of intracellualr spaces the cell-permanent ester (SNARF calcein AM) was injected. In both cases approximately 10 ml of dye solution (dye dissolved in 1:3 diluted sea water) was used. The experimental conditions were in principle, the same as described above, with the exception that normocapnia (normal levels of carbon dioxide) could not be maintained during anoxia (absence of oxygen), as gas-mixing pumps were not available at that time. But these experiments and the BCECF measurements at

		anoxia/normocapnia) yielded similar results. They have been included because they demonstrate clearly the advantages of optical methods to image systemic processes.

5.2	**Results and discussion**	Heart rate and pH changes at varying ambient P_{CO2} / pH were studied in *Daphnia magna*. Gas mixing pumps were utilised to modify P_{CO2} in the medium, resulting also in pH changes. Heart rate changes could not be provoked by P_{CO2} changes during normoxia (normal levels of oxygen). However, during severe hypoxia (approximately 3.6% oxygen), the application of hypercapnia (2% carbon dioxide, pH 6) caused a transient decrease of heart rate by 20-50 beats per minute. The minimum was reached within a few minutes. After 20 minutes, the heart had returned back to a rate similar to the pre-hypercapnic (normocapnic) value (pH 7.45). Switching then to normocapnia (normal levels of carbon dioxide) or acapnia (0% carbon dioxide, pH 8.5) caused the heart rate to either non-transiently or transiently to increase. Concerning blood pH, the application of hypercapnia (increased carbon dioxide) caused a transient decrease of pH even during normoxia (normal levels of oxygen). The minimum was reached within 10 minutes. After 20-30 minutes, the pH had increased to a stable value, which was a little lower than the pre-hypercapnic one. The pH measurements by optical methods in whole animals are novel, and should not yet be regarded as a final analysis of acid-base balance in *Daphnia*, but they give a first view on specific links between pH, heart activity and metabolism. The intracellular pH measured in the shell gland reflected the anaerobic metabolic activity. Lactate production is indirectly linked with proton release, and anoxia (absence of oxygen) caused intracellular pH to decrease. Due to protons released from the cells, the extracellular pH also dropped due to anoxia showing a time-course which is a little faster, but similar to the heart rate course. During early anoxia lactate and protons were generated at a high rate in cells and tissues, and they were released to the blood and also transported to the heart. The similarity of both time-courses, heart rate and pH indicates that heart activity (heart rate) is influenced or even controlled by pH. Steady changes in carbon dioxide partial pressure / pH caused a transient change in blood pH. *Daphnia magna* was obviously able to control pH during an ambient-causes respiratory acidosis. A steady change of ambient carbon dioxide partial pressure / pH caused a transient drop of heart rate (with a time course similar to blood pH) indicating again a functional relationship between heart rate and pH. In *Daphnia* the control of respiration depends on adaptive changes of heart rate. Apart from ambient oxygen partial pressure, internal carbon dioxide pressure and pH may also be input signals for respiratory control. It is known, in any case, that a decreasing extracellular or intracellular pH causes a depression of trans-sarcolemmal Ca^{2+} currents and contraction in cardiac muscle cells.
5.2.1	EC_0	Not reported.
5.2.2	EC_{50}	Not reported.
5.2.3	EC_{100}	Not reported.
5.3	**Conclusion**	Data reported in this study shows that *Daphnia magna* can tolerate carbon dioxide partial pressures of 2 % v/v both in the presence of

	normal levels of oxygen and severe hypoxia (3.6% oxygen). Note that author comments that *Daphnia* are known to be naturally exposed to ambient carbon dioxide concentrations of up to 2%.

5.3.1	Reliability	3
5.3.2	Deficiencies	Yes. This study was not carried out to EC Method C2 in Annex V of Directive 67/548/EEC. Rather than looking at acute toxicity *per se*, this report investigates heart rate and pH changes at varying ambient P_{CO2} / pH in *Daphnia magna*. This study shows that *Daphnia magna* can tolerate carbon dioxide partial pressures of 2 % v/v both in the presence of normal levels of oxygen and severe hypoxia (3.6% oxygen).

Table A7_1_1_2-1 Description of test organisms

Criteria	Details
Strain	*Daphnia magna* Straus
Source	Descended from a clone (clone 5) cultured at the University of Sheffield, Department of Animal and Plant Physiology, United Kingdom.
Age	Not reported, but in most experiments the animals had a body length of 2.5 +/- 0.2 mm (measured from the anterior most part of the head to the base of the apical spine), and a body mass of approximately 1 mg.
Breeding method	Descended from a clone (clone 5) cultured at the University of Sheffield, Department of Animal and Plant Physiology, United Kingdom.
Kind of food	Algae
Feeding frequency	Not fully described, other than "keeping and feeding of animals were identical to previous studies by the same author."
Pre-treatment	Animals were acclimated in the chamber for about 1h.
Feeding of animals during test	Not fully described, other than "keeping and feeding of animals were identical to previous studies by the same author."

Table A7_1_1_2-2 Description of test system

Criteria	Details
Renewal of test solution	A constant medium flow (8-10 ml min^{-1}) through the chamber was generated by a peristaltic pump located after it. The test solution (1:14 diluted seawater) was sucked up from a thermostated glass vessel with a small opening (transport timefrom vessel to chamber 6s), where it was equilibrated with different O2/N2 mixtures (at normocapnic conditions 400 ppm CO2), using gas mixing pumps.
Volume of test vessels	Not reported. Test vessels described as follows: An animal chamber made of anodized aluminium was utilised for the experiments. Two cover slides were used as top and bottom of the inner room, whose lateral wall was the inside of the water filled theromstated casing.
Volume/animal	Not reported.
Number of animals/vessel	One.
Number of vessels / concentration	Not reported.
Test performed in closed vessels due to significant volatility of TS	Yes.

Table A7_1_1_2-3 Description of test conditions

Criteria	Details
Test temperature	Temperature of the medium leaving the chamber (see table A7_1_1_2-2 renewal of test solution) was continually checked using a needle-shaped thermoelement (NiCr-Ni Sensortek, Clifton NJ)
Dissolved oxygen	Oxygen partial pressure of the medium leaving the chamber (see table A7_1_1_2-2 renewal of test solution) was continually checked using a small oxygen electrode (P/N SI 130, Strathkelvin Instruments Glasgow, UK).
pH	The transparency of *Daphnia magna* allowed the application of pH sensitive dyes to determine pH changes.
Adjustment of pH	No.
Aeration of dilution water	Not reported.
Quality / intensity of irradiation	Not reported.
Photoperiod	Not reported.

Section A7.4.1.2 **Acute toxicity to invertebrates (1 of 3)**
Annex Point IIA, VII, 7.2 Special investigation in *Daphnia magna*

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted.
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Materials and Methods	*State if applicants version is acceptable, or indicate relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*
Results and discussion	*Adopt applicant's version or include revised version. If necessary, discuss relevant deviations from applicant's view referring to the (sub)heading numbers.*
Conclusion	Other conclusions: *(adopt applicant's version or include revised version)*
Reliability	*Based on assessment of materials and methods include appropriate reliability indicator.*
Acceptability	acceptable / not acceptable *(give reasons if necessary e.g. if a study is considered acceptable despite a poor reliability indicator. Discuss the relevance of deficiencies and indicate if repeat if necessary).*
Remarks	
	COMMENTS FROM …..
Date	*Give date of comments submitted.*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion* *Discuss if deviating from view of rapporteur member state. .*
Results and discussion	*Discuss if deviating from view of rapporteur member state.*
Conclusion	*Discuss if deviating from view of rapporteur member state.*
Reliability	*Discuss if deviating from view of rapporteur member state.*
Acceptability	*Discuss if deviating from view of rapporteur member state.*
Remarks	

Section A7.4.1.2
Annex Point IIA, VII, 7.2

Acute toxicity to invertebrates (2 of 3)

Special investigation in Cladocera species *Daphnia similis* *Monia flagellata* and *Ceriodaphnia rigaudi*

Official use only

1. REFERENCE

Already submitted for carbon dioxide dossier for Product Type 14. Amendments made to the sections on specification and deficiencies.

1.1	**Reference**	[redacted]
1.2	**Data protection**	[redacted]
1.2.1	Data owner	[redacted]
1.2.2		
1.2.3	Criteria for data protection	[redacted]

2. GUIDELINES AND QUALITY ASSURANCE

2.1	**Guideline study**	No. Not carried out to EC Method C2 in Annex V of Directive 67/548/EEC.
2.2	**GLP**	No. GLP was not compulsory at the time the study was performed.
2.3	**Deviations**	Yes. No set guideline followed.

3. MATERIALS AND METHODS

3.1	**Test material**	[redacted]
3.1.1.	Lot/Batch number	[redacted]
3.1.2	Specification	[redacted]
		[redacted]
3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not applicable. No special procedures were applied to determine carbon dioxide content in water.
3.3	**Reference substance**	No.
3.3.1	Method of analysis for reference substance	Reference substance not used.

Section A7.4.1.2
Annex Point IIA, VII, 7.2

Acute toxicity to invertebrates (2 of 3)
Special investigation in Cladocera species *Daphnia similis* *Monia flagellata* and *Ceriodaphnia rigaudi*

3.4	**Testing procedure**	
3.4.1	Dilution water	Refer to table A7_1_1_2-1 for description of test site and sampling protocol
3.4.2	Test organisms	Abundance of cladocera in the test pond was used as indication of the effect of seasonal variation in carbon dioxide concentration. Species counted were *Daphnia similis* *Monia flagellata* *Ceriodaphnia rigaudi*
3.4.3	Test system	Refer to table A7_1_1_2-1 for description of test site and sampling protocol
3.4.4	Test conditions	Refer to table A7_1_1_2-1 for description of test site and sampling protocol
3.4.5	Duration of test	12 months (measurements taken from July 1980 to June 1981).
3.4.6	Test parameter	Number of individuals present in test pond.
3.4.7	Sampling	Fortnightly. Refer to table A7_1_1_2-1 for description of test site and sampling protocol.
3.4.8	Monitoring of TS concentration	Monthly.
3.4.9	Statistics	Not reported.
		4 RESULTS
4.1	**Limit Test**	Not performed.
4.2	**Results test substance**	
4.2.1	Initial concentrations of test substance	Carbon dioxide fluctuated from almost nil to 86 mg/L during the period of investigation.
4.2.2	Actual concentrations of test substance	Carbon dioxide fluctuated from almost nil to 86 mg/L during the period of investigation.
4.2.3	Effect data (Immobilisation)	Carbon dioxide did not seem to be the conditioning factor for the production and growth of Cladocera. *Daphnia similis* not only occurred at carbon dioxide concentrations of 86 mg/L but registered a peak showing that they can survive in high carbon dioxide concentrations. *Monia flagellata* and *Ceriodaphnia rigaudi* showed peaks at carbon dioxide concentrations of 12 and 32.6 mg/L respectively.
4.2.4	Concentration/ response curve	Not reported.
4.2.5	Other effects	None reported.
4.3	**Results of controls**	No control species reported.
4.4	**Test with reference substance**	Not performed.

Section A7.4.1.2 **Acute toxicity to invertebrates (2 of 3)**

Annex Point IIA, VII, 7.2 Special investigation in Cladocera species *Daphnia similis* *Monia flagellata* and *Ceriodaphnia rigaudi*

5 APPLICANT'S SUMMARY AND CONCLUSION

5.1 Materials and Methods

This study was not carried out to EC Method C2 in Annex V of Directive 67/548/EEC.

Water samples were collected from a pond, about 24 km from Ludhiana at Village Raqba (Ludhiana) situated at the link road connecting Boparai to Mullanpur. (India).

The water level in pond varied from 20 cm in June to 40-50 cm in July – September. The water was muddy during June-July, greenish during August-September and transparent during February-March. The pH ranged between 7.20 (July) and 9.75 (December). The sample collections were made at fortnightly intervals by sieving 100 litres of water through a plankton net (300 meshes per square cm), from three marked points (A, B and C) at the peripheral regions of the pond. The material caught was preserved in 10% formalin. In the laboratory, the numerical estimation of population size was carried out according to Welch (1952).

The water temperature, Secchi Disc Transparency, Colour and water depth were all measured before collecting the material. Dissolved oxygen, pH and carbon dioxide were estimated using standard methods for the examination of water and waste water (14th ed. Amer. Public Health Assoc Inc, New York).

5.2 Results and discussion

The test pond harboured insects, fish, frogs and tortoises, besides plankton. The zooplankton largely comprised of Cladocera, Copepoda and Rotifera. Cladocerans were represented by six species in all, of these *Monia flagellata* was noted in maximum number of 15 August 1980 when its actual number was counted 1827 individuals per litre. This species was not found in the test pond from 15 November 1980 to 15 May 1981 when only 2 individuals per litre occurred. *Ceriodaphnia rigaudi* was found in the pond from 15 August 1980 to 15 May 1981 reaching maximum number on 15 September 1980. *Daphnia similis* was the third species found in abundance and occurred in the pond from 1 November 1980 to 15 May 1981, but was absent on 15 December 1980. The maximum pulse of this species was recorded on 15 January 1981, when its number reached 421 individuals per litre.

5.2.1 EC_0 Not reported.

5.2.2 EC_{50} Not reported.

5.2.3 EC_{100} Not reported.

5.3 Conclusion

Data reported in this study shows that *Daphnia similis* not only occurred at carbon dioxide concentrations of 86 mg/L but registered a peak showing that they can survive in high carbon dioxide concentrations. *Monia flagellata* and *Ceriodaphnia rigaudi* showed peaks at carbon dioxide concentrations of 12 and 32.6 mg/L respectively.

5.3.1 Reliability 3

5.3.2 Deficiencies Yes.

This study was not carried out to EC Method C2 in Annex V of Directive 67/548/EEC.

Rather than looking at acute toxicity *per se*, this report measures the natural fluctuations in background carbon dioxide concentrations in a pond, over 12 months and its effect on the survival of the Cladocera species *Daphnia similis* *Monia flagellata* and *Ceriodaphnia rigaudi.*

Table A7_1_1_2-1 Description of test site and sampling protocol

Criteria	
Description of site	Water samples were collected from a pond, about 24 km from Ludhiana at Village Raqba (Ludhiana) situated at the link road connecting Boparai to Mullanpur. (India)
Description of test pond : Appearance of water	Water level in pond varied from 20 cm in June to 40-50 cm in July – September. The water was muddy during June-July, greenish during August-September and transparent during February-March.
Description of test pond : pH of water	pH ranged between 7.20 (July) and 9.75 (December).
Number of sampling sites	Three marked points (A, B and C) at the peripheral regions of the pond.
Sample intervals	Fortnightly
Sample preparation	100 litres of water sieved through a plankton net (300 meshes per square cm), and material caught was preserved in 10% formalin.

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted.
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Materials and Methods	*State if applicants version is acceptable, or indicate relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*
Results and discussion	*Adopt applicant's version or include revised version. If necessary, discuss relevant deviations from applicant's view referring to the (sub)heading numbers.*
Conclusion	Other conclusions: *(adopt applicant's version or include revised version)*
Reliability	*Based on assessment of materials and methods include appropriate reliability indicator.*
Acceptability	acceptable / not acceptable *(give reasons if necessary e.g. if a study is considered acceptable despite a poor reliability indicator. Discuss the relevance of deficiencies and indicate if repeat if necessary).*
Remarks	
	COMMENTS FROM …..
Date	*Give date of comments submitted.*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion* *Discuss if deviating from view of rapporteur member state. .*
Results and discussion	*Discuss if deviating from view of rapporteur member state.*
Conclusion	*Discuss if deviating from view of rapporteur member state.*
Reliability	*Discuss if deviating from view of rapporteur member state.*
Acceptability	*Discuss if deviating from view of rapporteur member state.*
Remarks	

Section A7.4.1.2 **Acute toxicity to invertebrates (3 of 3)**
Annex Point IIA, VII, 7.2 Special investigation in ants

Official use only

1. REFERENCE

Already submitted for carbon dioxide dossier for Product Type 14. Amendments made to the sections on specification and deficiencies.

1.1 Reference [redacted]

1.2 Data protection [redacted]

1.2.1 Data owner [redacted]

1.2.2

1.2.3 Criteria for data protection [redacted]

2. GUIDELINES AND QUALITY ASSURANCE

2.1 Guideline study No.
Not carried out to EC Method C2 in Annex V of Directive 67/548/EEC.

2.2 GLP No.
No record of study being conducted in compliance with the requirements of GLP.

2.3 Deviations Yes.
No set guideline followed.

3. MATERIALS AND METHODS

3.1 Test material [redacted]

3.1.1. Lot/Batch number [redacted]

3.1.2 Specification [redacted]

[redacted]

Section A7.4.1.2 **Acute toxicity to invertebrates (3 of 3)**
Annex Point IIA, VII, 7.2 Special investigation in ants

3.2	**Preparation of TS solution for poorly soluble or volatile test substances**	Not applicable. No special procedures were applied to determine carbon dioxide content in water.
3.3	**Reference substance**	No.
3.3.1	Method of analysis for reference substance	Reference substance not used.
3.4	**Testing procedure**	
3.4.1	Dilution water	Refer to table A7_1_1_2-1 for description of test site.
3.4.2	Test organisms	Mangrove ant *Polyrhachis sokolova*
3.4.3	Test system	Refer to table A7_1_1_2-1, at the end of this study summary for description of test site. Nest concentrations of carbon dioxide were determined using air probes at different depths of the nest of the mangrove ant.
3.4.4	Test conditions	The nests of the mangrove ant *Polyrhachis sokolova* are found in soil in intertidal mangrove communities, and thus are covered in water at high tide for several hours. Some of the nest galleries are flooded, but others retain air pockets where the ants retreat. During and following inundation (where the ants nests were covered in water), carbon dioxide concentrations in the ants nest were measured from air samples collected from different levels in the nest, and from artificial control "holes" in the mud away from the nest. This study measured normal, natural background levels of carbon dioxide in an ant's nest. Refer to table A7_1_1_2-1, at the end of this study summary for description of test site.
3.4.5	Duration of test	11 days.
3.4.6	Test parameter	Carbon dioxide concentration in the ants nest.
3.4.7	Sampling	Gas samples were drawn from the nest chambers while the nest was under water (0 day), 1h after the water receded and then 1,2, 5, 8 and 11 days after the nest was covered in water (referred to in study report as "inundation"). Control measurements, taken from artificial holes in the mud away from the ants' nest were taken on day 5, 6, 8 and 11 after the mud was covered in water.
3.4.8	Monitoring of TS concentration	Refer to 3.4.7 Sampling (above) for details.
3.4.9	Statistics	A Kruskal-Wallis Test was used to compare carbon dioxide concentrations at different depths and from different times in the tidal cycle (time since nest was covered in water, referred to in study report as "inundation") in the nest chambers and from different times in the artificial (control) holes. A Mann-Whitney *U*-test was used to compare the carbon dioxide concentrations in the control holes at shallow (<10 cm) and deep (> 10cm) levels. A probability level of 0.05 was used to determine statistical significance.

Section A7.4.1.2 **Acute toxicity to invertebrates (3 of 3)**
Annex Point IIA, VII, 7.2 Special investigation in ants

		4 RESULTS
4.1	**Limit Test**	Not performed.
4.2	**Results test substance**	
4.2.1	Initial concentrations of test substance	Nest carbon dioxide concentrations were high (2.5% - 11%) during and immediately after the nest was covered in water (referred to in study report as "inundation"), but the carbon dioxide concentration in the upper regions of the nest fell as soil water levels receded. However, at depths >10 cm below the level soil surface the carbon dioxide concentrations remained relatively high and stable (at approximately 2%) over the 11 day test period (between one high tide and the next).
4.2.2	Actual concentrations of test substance	Refer to "4.2.1 Initial concentrations of test substance" (above) for details.
4.2.3	Effect data (Immobilisation)	The carbon dioxide concentrations in the nests of the mangrove ant *Polyrhachis sokolova* during high tides are among the highest recorded for insect nests, suggesting that these ants may have unusual physiological attributes to match the behavioural and ecological challenges associated with living in an intertidal zone.
4.2.4	Concentration/ response curve	Not reported.
4.2.5	Other effects	None reported.
4.3	**Results of controls**	Air samples collected from artificial control "holes" in the mud away from the nest. Refer to table A7.1.1.2-3 at the end of this study summary for details of control measurements.
4.4	**Test with reference substance**	Not performed.
		5 APPLICANT'S SUMMARY AND CONCLUSION
5.1	**Materials and Methods**	This study was not carried out to EC Method C2 in Annex V of Directive 67/548/EEC. For details of study sites, see A7_1_1_2-1 at the end of this study summary. <u>Determination of nest concentrations of carbon dioxide</u> At site 1 (Rapid Creek) 10 air probes were placed in the two nests so that air samples could be extracted from different depths of the nest. The probes consisted of a 30 cm long plastic pipette (1 mL) with an injection needle glued into the upper end, so that a three-way valve could be placed at the top. All probes were placed with their tip into a nest chamber and a layer of plaster of paris was placed on the soil surface around the probe to prevent water intrusion. Gas samples were drawn from the nest chambers while the nest was under water (0 days since inundation), 1h after the water receded and then 1,2, 5, 8 and 11 days after the water had receded (inundation). Thus, the gaseous environments in the nests were sampled between one high tide and the next (when the nest was covered in water). In addition to the permanent probes at site 1, a portable probe was used at site 2 (East Arm, Darwin Harbour) to take gas samples from the nest when it was under water (during inundation). The portable probe was inserted into the mud until it struck an ant gallery, then the sample was drawn. (continued....)

5.1 Materials and Methods

(Continued)

Control samples: Determination of carbon dioxide in soil

To determine, independently from the ants, the effect of depth in the soil and time since the last high tide when the mud was covered with water (inundation) on carbon dioxide concentrations, six artificial control holes with a diameter of 2-3 cm and depths from 7-25 cm were made at site 1 (Rapid Creek). Permanent probes were placed inside the holes, and the top of the holes were sealed with mud and plaster of paris.

The samples of air from the nest chambers and the control holes were withdrawn with a 5 ml glass syringe. By using a second syringe and the three way valve, the probe and the "dead volume" in the syringe was flushed with air from the nest before the sample was taken. The tubes were flushed twice with 2 mL of air and then an air sample of 4-5 mL was drawn. The syringe with the closed three-way valve was removed from the probe and a new three-way valve was placed on the probe.

Sources of carbon dioxide in the nest: Contribution of Ant Respiration to Carbon Dioxide Concentration in Nest

To estimate the contribution of ant respiration to the carbon dioxide concentration of the nests, carbon dioxide production of individual ants and number of ants living in the nest was measured. The carbon dioxide production of the brood was measured at 25°C after placing two broods in the experimental chamber at a time (n= 5 sets of larvae, n = 2 sets of pupae). The respiration chambers were cylindrical glass tubes with a length of 60 mm and a diameter of 13 mm with both ends stopped y rubber plugs. The chambers were placed in a temperature regulated water baths during the measurements. The number of workers was estimated for the two nests at site 1 using a mark-recapture technique. The workers were captured using a small portable vacuum cleaner and trapped in a bag made of nylon mesh, which allowed marking of their gaster with white correction ink directly through the mesh without anaesthetising the ants. The ants were captured when leaving the nest entrances, marked and released within 30 min. Ants were recaptured the following day when workers were leaving the nest.

Sources of carbon dioxide in the nest: Contribution of Microbial Respiration in the mud

To assess the contribution of microbial respiration in the mud, independently of the ants, respiration was measured from sediment (mud) samples taken during the construction of the control holes at depths of 0, 2, 7 and 20 cm, and from nests at depths of 5 and 15 cm. The samples were taken horizontally in the holes with a 6 mL plastic syringe (with the tapered end cut off), and immediately sealed with an airtight plastic cap. In the laboratory, the first 1cm of the soil core was removed and then 1mL was taken and placed in the respiration chamber. The respiratory measurements were taken at 25°C.

Determination of Carbon Dioxide in Samples

The air samples from the nests and the respiratory measurements were analysed for carbon dioxide using a flow-through analyser model LI-6251, connected to a data acquisition and analyser system (Sabel System International, Nevada USA, using Datacan V software). The airflow was kept constant at 150 mL per minute.

(continued....)

5.1	**Materials and Methods** (Continued)	Determination of Gallery Volumes in the Nest At the end of the experimental period, the volume of the galleries in Nest A at site 1 (Rapid Creek) was determined by removing two soil cores (2 cm in diameter and 30 cm long) from the centre of the nest, so that galleries in all depths were exposed to the holes. A measured amount of water was added through two funnels until the water level reached the top of the nest.
5.2	**Results and discussion**	Nest concentrations of carbon dioxide Nest concentrations of carbon dioxide in the deeper parts of the *Polyrhachis sokolova* nest fluctuated between 2 and 3%, and although the mean concentrations were generally higher than other parts of the nest, they were only statistically higher during days 0 and 5 after the nest was covered in water (inundation). The carbon dioxide concentration in the middle level (<10 cm below the soil surface) was significantly higher than in the nest mound 1h after the water receded, but by the next day there was no significant difference among the depths. In the nest mound, the carbon dioxide concentration at 0 days (when the nest was covered in water) was significantly greater than all the other sample periods. At the middle level (<10 cm below soil surface) the carbon dioxide concentration was higher 1h after the water receded compared to all the other time periods. The carbon dioxide concentrations at the deep levels in the nest were not significantly different across all time periods from day 0 (when the nest was covered in water) to 11 days after the water receded. Air samples from the nest at site 2 (East Arm, Darwin Harbour) showed a very high carbon dioxide concentration when it was covered in water, with values up to 11% at depths below 10 cm and 8.7% at depths between 0 and 10cm. One hour after 3h of being covered in water, the carbon dioxide concentration had decreased to 2.2% and 0.8% at the respective depths. By using a portable probe to sample the various ant galleries when the nest was covered in water, it was found that although some galleries were still full of air, others filled with water and this was the case at all depths in the nest. Thus, the nest was not completely water tight and the air volume available to the ants decreased when the nest was covered in water. The high carbon dioxide concentration in the control holes shows that soil respiration is quite significant. As the tide encroaches upon the nest the water level rises from below and at the same time tidal water infiltrates the nest from above. Therefore, carbon dioxide rich air from the soil capillary space will be replaced by water and some of the air will end up in the galleries and be trapped in the air pockets. Thus, the air in the air pockets will reach very high concentrations of carbon dioxide due to the combined effects of the high density of ants and the input of carbon dioxide from the soil. The carbon dioxide concentrations in the nests were most variable at the deepest level, and at all levels when the nest was covered in water. During inundation, the ants aggregated in galleries that are presumably segregated from other galleries due to the flooding of some parts of the nest and these clumps of ants may account for the variability in carbon dioxide at this time. (Continued)

5.2 Results and discussion (continued)

Soon after the water recedes from the nest mound and the middle of the nest < 10 cm deep), the carbon dioxide concentrations fall. The carbon dioxide concentrations are highest in the middle region of the nest 1h after the water has receded, because there is still water in the soil (even though the mound has already drained), and because the nest has been covered in water for at least an hour longer (compared to time 0). The concentrations in the middle region of the nest at this time are similar to those in the deeper galleries when the nest is covered in water. No deep galleries were sampled 1h or 1 day after the nest was covered in water, because all the deep galleries being sampled were full of water. When the water level falls in the soil, fresh atmospheric air is drawn in to the nest galleries through the four nest openings, diluting the air in the upper parts of the nest. This process presumably continues until the water level falls below the deepest galleries in the nest. The carbon dioxide production from the ants and from the mud continually adds carbon dioxide to the nest. Therefore the resulting carbon dioxide concentration depends on the two opposing processes: input of fresh air as the water level falls, and the production of carbon dioxide by ants and mud.

The main factor in the ventilation of the nests of *Polyrhachis sokolova* is the changing water level inside the nest, and without this flushing of air the carbon dioxide concentration in the nest would reach very high levels due to the intense biological activity in the mud. After exposure to fresh air after the water receded, the upper parts of the nest reached equilibrium by the first day after the nest was covered in water and the carbon dioxide concentration remained relatively stable until the next high tide. The deepest parts of the nest experienced a stable carbon dioxide concentration throughout the tidal cycle presumably because deep galleries were less diluted by fresh air and nay influx of air caused by falling water levels was from parts of the nest above, rather than atmospheric air.

The extremely high concentrations of carbon dioxide in the nest at site 2 (East Arm, Darwin Harbour) may be due to the location of this site deep within the tidal zone, resulting in a higher tide and longer period of being covered with water. Also the nest at East Arm, Darwin Harbour had no mound so it did not drain as quickly or as often as the nests in site 1 (Rapid Creek).

<u>Carbon dioxide concentrations in the control samples</u>
For results of measurements taken to determine the carbon dioxide concentration in mangrove mud, refer to table A7.1.1.2-3 at the end of this study summary.

The mean carbon dioxide concentrations in the control holes in the soil near the nests were generally higher at deeper depths (>10 cm), but the difference was only statistically significant 11 days after inundation. The carbon dioxide concentrations did not differ across the time periods in the shallow holes, but in the deeper holes (>10 cm), there was a significant decrease in carbon dioxide concentrations that occurred between days 6 and 8 inundation.

(continued...)

5.2	**Results and discussion (continued)**	Sources of carbon dioxide For results of measurements taken to determine the sources of carbon dioxide in the nest e.g. due to respiration of the ants, see table A7_1_1_2-2 at the end of this study summary. Estimates of population density in ant colonies by capture-release-recapture techniques are always encumbered with sources of error due to the lack of complete mixing of marked individuals in the nest. The volume of nest A at site 1 (Rapid Creek) was estimated as 64 L, but it is acknowledged that some water could have leaked during the filing procedure and, on the other hand, some galleries could still have contained air pockets. The carbon dioxide production from ants increased the total carbon dioxide concentration by (50 x 1000/640000) 0.008% per hour. Assuming that the mean diameter of the galleries is 2cm, 1L of galleries has a surface area of 1000 cm^2 giving a total gallery surface of 64000 cm^2. The carbon dioxide input from mud is (6.2 x 64000 µL/h) 396 mL/h equivalent to an increase in the total nest concentration of 0.6% per hour. Thus the ants only add approximately 10-15% of the total carbon dioxide to the nest, which is consistent with the high values of carbon dioxide found in the control holes. During inundation the air volume available to the ants decreases and the density of the ants in the air pockets is at it's highest and under these conditions the ants may contribute much more to the increase of carbon dioxide concentration.
5.2.1	EC_0	Not reported.
5.2.2	EC_{50}	Not reported.
5.2.3	EC_{100}	Not reported.
5.3	**Conclusion**	Results to this test show that nest carbon dioxide concentrations were high (2.5% - 11%) during and immediately after the nest was covered in water (referred to in study report as "inundation"), but the carbon dioxide concentration in the upper regions of the nest fell as soil water levels receded. However, at depths >10 cm below the level soil surface the carbon dioxide concentrations remained relatively high and stable (at approximately 2%) over the 11 day test period (between one high tide and the next). Carbon dioxide concentrations in muds around the nest can fluctuate between 0.5% and 2% < 10cm below the surface and between 1.2% and 3.% > 10 cm below the surface. Soil microbial respiration is a major source of carbon dioxide in the nests of *Polyrhachis sokolova*, but if large numbers of ants are restricted to relatively small portions of the nest (e.g. during inundation), then ant respiration is a mjor contributor to carbon dioxide concentrations during high tides.
5.3.1	Reliability	3
5.3.2	Deficiencies	Yes. This study was not carried out to EC Method C2 in Annex V of Directive 67/548/EEC. Rather than looking at acute toxicity *per se*, this report measures the natural fluctuations in background carbon dioxide concentrations in a the nests of the mangrove ant, *Polyrhachis sokolova*.

Table A7_1_1_2-1 Description of test site

Criteria	
Description of site	The study was performed in the mangroves around Darwin, NT Australia (12°30'S 131°00E), in the dry season of 2002. Site 1 The main experimental site (site 1) was established on the east bank of Rapid Creek, approximately 150m from the creek and 600 m from the sea neighbouring the Charles Darwin University, and included two nests, labelled A and B. The site is very well protected, vegetated by 4-5 m tall trees of *Rhizophora stylosa* and *Avicennia marina*. Site 2 This site was located at East Arm in Darwin Harbour near the former World War II quarrantine area. The site is only 100m from the harbour and is quite exposed, so waves can often occur during inundation. The vegetation is a pure stand of *Rhizophora stylosa* 5-7 m tall.
Description of test nests.	General description of mangrove ants nest Nests of *Polyrhachis sokolova* sometimes include a central mound elevated above the surrounding soil surface. The size and shape of the mounds were measured at the two sites, and elevations were calculated by measuring the height of the water level above the nest at maximum tide, where the height above the lowest astronomical tide (LAT) is tabulated for the Darwin area. Site 1 The surface is very muddy with many crab holes and piles of excavated mud from the ants' digging activity. The elevation of the nests at this site (Rapid Creek) were 6.75 cm above lowest astronomical tide (LAT) for nest A, and 6.65 cm above LAT for nest B. This means that nest B was inundated during 27% of the high tides with up to 1.35m of seawater. The mound of nest A has a diameter of 100 cm and a height 20-25 cm above the surroundings, whereas the mound of nest B only reached 17 cm and the diameter was 80 cm. Both nests had four entrances. Site 2 The substrate is sandy on the surface but very muddy below 1-2 cm. There is very little sign of crab activity, so the surface was smooth. The nest at this site (East Arm) was 5.85 cm above lowest astronomical tide (LAT), thus it was inundated by more than 2.15 m of seawater during more than 64% of high tides. The nest had no mound, but there were volcano-like walls around the two entrances 4-5 cm high.

Table A7_1_1_2-2 Sources of carbon dioxide in the nests at site 1 (Rapid Creek)

Criteria			
The specific respiratory rates +/- SD for individuals workers of *Polyrhachis sokolova* *	At 15°C At 25°C At 35°C	1.45 +/-0.02 µL CO2/mg dry weight/h 2.79 +/-0.74 µL CO2/mg dry weight/h 4.27 +/- 0.94 µL CO2/mg dry weight/h	
The specific respiratory rates +/- SD for larvae and pupae of *Polyrhachis sokolova*	At 25°C	Larvae 0.50 +/- 0.06 µL CO2 /mg dry weight/h Pupae 0.56 +/- 0.04 µL CO2/mg dry weight/h	
The mean (+/- SD) fresh and dry weights for workers (n = 10)		Fresh weight : 17.7 +/- 2.3 mg Dry weight: 5.9 +/- 0.8 mg	
The crude measurements of mud respiration from the nest		23.1+/- 7.3 µL CO2/mg fresh weight/h (or 6.2 +/- 2.0 µL CO2/ cm^2 surface of sample per hour).	
The population estimates of foraging workers at site 1		1051 +/-166 individuals in nest A 2065 +/- 463 individuals in nest B.	
The measured volume of galleries in nest A		64 L.	

Footnote: The population estimates apply only to the foraging population which can differ strongly from the total population. No information is available on the foraging behaviour of *Polyrhachis sokolova* so, for the calculations cited below, it is assumed that half the population is foraging. The total respiratory rate at 25°C by workers in nest A (site 1 Rapid Creek) can be calculated as (2 x 1050 x 5.9 x 2.8 µL/h) 34.7 mL/h. Assuming that the brood respiration is half the worker population the total carbon dioxide production in the nest would be approximately 50 mL h^{-1} at 25°C and approximately 100 mL h^{-1} at 35°C.

Table A7_1_1_2-3 Carbon dioxide concentration in mangrove mud (artificial holes, acting as control measurements)

Days since mud was covered with water (inundation)	Concentration of carbon dioxide measured in mud	
	Shallow hole (< 10 cm)	Deep hole (> 10cm)
5	ca. 2.1%	3.5 %
6	ca. 2.3%	3.3%
8	0.5%	1.4%
11	0.5%	1.2%

Evaluation by Competent Authorities	
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted.
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Materials and Methods	*State if applicants version is acceptable, or indicate relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion.*
Results and discussion	*Adopt applicant's version or include revised version. If necessary, discuss relevant deviations from applicant's view referring to the (sub)heading numbers.*
Conclusion	Other conclusions: *(adopt applicant's version or include revised version)*
Reliability	*Based on assessment of materials and methods include appropriate reliability indicator.*
Acceptability	acceptable / not acceptable *(give reasons if necessary e.g. if a study is considered acceptable despite a poor reliability indicator. Discuss the relevance of deficiencies and indicate if repeat if necessary).*
Remarks	
	COMMENTS FROM …..
Date	*Give date of comments submitted.*
Materials and Methods	*Discuss additional relevant discrepancies referring to the (sub)heading numbers and to applicant's summary and conclusion* *Discuss if deviating from view of rapporteur member state. .*
Results and discussion	*Discuss if deviating from view of rapporteur member state.*
Conclusion	*Discuss if deviating from view of rapporteur member state.*
Reliability	*Discuss if deviating from view of rapporteur member state.*
Acceptability	*Discuss if deviating from view of rapporteur member state.*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.4.1.3 **Annex Point/TNsG** **Annex IIA, VII.7.3**	**Growth inhibition test on algae** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [ ]	**Technically not feasible** [ ] **Scientifically unjustified** [✓]	
Limited exposure [ ]	**Other justification** [ ]	
Detailed justification:	It is not scientifically necessary to calculate the growth inhibition of CO_2 to algae because CO_2 is an essential substrate for photosynthesis. [redacted]	
Undertaking of intended data submission [ ]	Not applicable	

Section 7.4.1.3 Annex Point/TNsG Annex IIA, VII.7.3	**Growth inhibition test on algae** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FROM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	

Table 4-2: Standard form for justification of the non-submission of data

Section 7.4.1.4 **Annex Point/TNsG Annex IIA, VII.7.4, Annex IIIA, VII.3**	**Inhibition to microbiological activity** Section 7: Ecotoxicological Profile, including Fate and Behaviour	
	JUSTIFICATION FOR NON-SUBMISSION OF DATA *As outlined in the TNsG on data requirements, the applicant must always be able to justify the suggested exemptions from the data requirements. The justifications are to be included in the respective location (section) of the dossier.* *If one of the following reasons is marked, detailed justification has to be given below. General arguments are not acceptable*	Official use only
Other existing data [✓]	**Technically not feasible** [ ] **Scientifically unjustified** [ ]	
Limited exposure [ ]	**Other justification** [ ]	
Detailed justification:	Whilst elevated levels of dissolved CO_2 may affect environmental conditions for bacteria by reducing pH, there are a number of mitigating factors that would reduce any environmental impacts of such changes and which make it unnecessary to generate new test data. a) Most free-living prokaryotic bacteria can tolerate a pH range of about 3 units (three orders of magnitude changes in H^{+} [1]. b) There is a high level of functional redundancy amongst mixed communities of micro-organisms such that declines in population of some species e.g. due to unfavourable pH conditions, will be compensated for by increases in others. The effect of this biological diversity and different environmental optima for different species means that bacteria can live in a wide range of pH conditions, from 0.5 – 9.0[1]. [redacted]	
Undertaking of intended data submission [ ]	Not applicable	

Section 7.4.1.4 Annex Point/TNsG Annex IIA, VII.7.4, Annex IIIA, VII.3	**Inhibition to microbiological activity** Section 7: Ecotoxicological Profile, including Fate and Behaviour

	Evaluation by Competent Authorities
	Use separate "evaluation boxes" to provide transparency as to the comments and views submitted
	EVALUATION BY RAPPORTEUR MEMBER STATE
Date	*Give date of action*
Evaluation of applicant's justification	*Discuss applicant's justification and, if applicable, deviating view*
Conclusion	*Indicate whether applicant's justification is acceptable or not. If unacceptable because of the reasons discussed above, indicate which action will be required, e.g. submission of specific test/study data*
Remarks	
	COMMENTS FROM OTHER MEMBER STATES *(specify)*
Date	*Give date of comments submitted*
Evaluation of applicant's justification	*Discuss if deviating from view of rapporteur member state*
Conclusion	*Discuss if deviating from view of rapporteur member state*
Remarks	