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BIODEGRADABILITY OF THE EEC RINGTEST CHEMICALS IN SEA WATER.

Comparison of a shake flask die-away test and the repetitive die-away test.

Ir. R. van den Berg, J.M.P. Buntsma-Hamers, N. Scholtz and B. Verboom

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0 SUMMARY

To contribute to a further development in sea water biodegradation testmethodology the EEC marine ringtest testchemicals have been studied in two modified biodegradability tests: the shake flask die-away test and the repetitive die-away test. The results of the shake flask die-away test and the repetitive die-away test agreed well.
The testsubstances aniline and sodiumbenzoate were found "readily biodegradable". Diethylene glycol also was degraded but did not met the pass criteria. Penta-erythritol and 4-nitrophenol were not degraded in any of the testseries.
In comparison with the shake flask die-away test the RDA test is preferred because of its many advantages, in spite of its more time-comsuming design.
Omittance of the nitrogen and phoporus nutrients decreased the degradation rate and level and delayed the start of the degradation.
Several improvements in the test procedure are suggested based on the results obtained.

1 INTRODUCTION

Annex V of Directive 79/831/EEC on Dangerous Chemicals specifies the methods to be used for the ecotoxicological evaluation of new chemicals. For the base-set information on biodegradability five screening-methods have been recommended by the OECD Expert Group on Degradation/Accumulation (OECD 1979). The guidelines of these methods have been given in annex VII of the Directive (84/449/EEC).
Information about biodegradability in sea water is of fundamental importance for the environmental hazard assessment. Until now only limited experience has been gained on biodegradability testing in sea water and it is uncertain to what extent the results of freshwater biodegradation tests can be used to predict the fate of organic compounds in sea water. An inter-laboratory comparison programme has been started by the EEC to evaluate how the described screeningtests, adapted to the use of sea water, might be used to determine ready biodegradability in sea water.
The purpose of this research was to contribute to a further development in sea water biodegradation testmethodology. Therefore two modified fresh water systems have been used: the shake flask die-away test and the repetitive die-away (RDA) test. We have had experiences for several years now with these two methods in fresh water testing. The shake flask die-away test was carried out in sea water with and without the addition of nitrogen and phosphorus nutrients. The RDA test in sea water was performed according to the standard procedure and in view of the EEC ringtest with "screeningtests" also with repetitive additions postponed for at least four weeks.

As testsubstances the compounds supplied for the EEC ringtest have been used. Aniline and sodiumbenzoate have been chosen as "readily biodegradable" compounds, diethylene glycol as a slowly degrading chemical and 4-nitrophenol and penta-erythritol as "erratic" substances.

The results of the mandatory shake flask die-away test and the RDA test with delayed repetitive additions have been send in for our participation in the EEC ringtest.

2 METHODS AND MATERIALS

2.1 Principles of the testmethods.

2.1.1 Shake flask die-away test.

With this method non volatile, water soluble organic compounds, present as only carbon source, are tested for their aerobic biodegradability in sea water. The degradation is followed by the analysis of the sumparameter dissolved organic carbon (DOC). The initial testconcentration is chosen depending the accuracy of the analysis, the dissolved organic carbon concentration of the medium and the toxicity of the testcompound. Generally concentrations of 10-40 g m^{-3} DOC are used.
A draft guideline has been described for the EEC ringtest (VKI 1985), adopted after EEC testmethod no C.3: The modified OECD screening test (EEC 1984a).

2.1.2 Repetitive die-away (RDA) test.

This method (Blok 1979) establishes the degradation by measurement of oxygen uptake and of the dissolved organic carbon. These two analytical procedures should confirm each other results. Besides water soluble organic compounds this method is als suitable for poorly water soluble and even volatile chemicals. Gerike (1984) recommends this method in a review on testing of poorly water soluble chemicals.

To the testprinciples of the shake flask die-away test and the Closed Bottle Test (EEC 1984b) two aspects have been added.

1. The presence of a reservoir of oxygen in the form of an air-bubble in the testbottle, makes it possible to test higher concentrations of testsubstances than in the Closed Bottle Test. The oxygen uptake is established by measuring the dissolved oxygen assuming an equilibrium of oxygen between the liquid- and the gasphase.
2. After a significant degradation (>10%) of the testcompound has been established, three repetitive additions of the testsubstance are performed. This increases the statistical value of the final result and provides information on the kinetics of the degradation (Blok and Booy 1984). Kinetic information is needed for prediction of the environmental fate of organic compounds.

The RDA guideline (Blok 1983) was changed with reference to the changes in the shake flask die-away test.
The statistical value of the degradation percentages determined in sea water is somewhat lower than of those determined in fresh water because the saturation concentration of oxygen in sea water (7.65 g m^{-3} at 20 °C and a salinity of 32.5 g kg^{-1}) is lower in comparison with freshwater (9.2 g m^{-3} at 20 °C)

2.2 Reagents and materials.

2.2.1 Sea water.

Freshly collected sea water was filtered and used as such for preparation of the testmedium and the plate count medium. The characteristics of the sea water are given in table 1.

2.2.2 Testmedium.

The testmedium was prepared by adding to the filtered sea water 1 ml per litre of the following solutions:

solution A:	KH_2PO_4	8.5 kg m^{-3}
	K_2HPO_4	21.75 kg m^{-3}
	$Na_2HPO_4.2\ H_2O$	33.3 kg m^{-3}
	NH_4Cl	1.07 kg m^{-3}
solution B:	$MgSO_4.7\ H_2O$	22.5 kg m^{-3}
solution C:	$CaCl_2$	27.5 kg m^{-3}
solution D:	$FeCl_3.6\ H_2O$	0.25 kg m^{-3}.

The solutions A to D had been sterilized by filtration (0.2 m). Only for the shake flask die-away test without NP nutrients, solution A was omitted.

2.2.3 Plate count medium.

The medium for the count of the number of bacteria had the following composition: yeast extract 2.5 kg, peptone 2.5 kg and agar agar 15.0 kg m^{-3} of filtered sea water. It was sterilized for 15 minutes at 121 °C.

2.2.4 Stocksolutions of the testsubstances.

The stocksolutions of the testsubstances have been prepared in distilled water. The theoretical stock-concentrations and initial testconcentrations are given table 2 together with the measured DOC.

2.3 Testprocedure.

2.3.1 Shake flask die-away test.

The test was performed in duplicate in 500 ml flasks with 200 ml of medium. Except for the blank, to this medium 1 ml of the stocksolution of the testchemicals was added. The flasks have been sampled weekly (5 ml of sample). After centrifugation (5000 x g) the samples were kept frozen at -15 °C until analysis. The flasks were incubated in the dark in a shaking incubator (150 rpm) at room temperature (18.0-21.4 °C). The testduration was extended from 4 (standard procedure screening test) to 11 weeks.

2.3.2 Repetitive die-away (RDA) test.

The test was carried out in triplicate in brown bottles of ± 280 ml volume with stirrers provided and ground glass stoppers. The bottles were filled with 186 ml of testmedium and 0.94 ml of the stocksolution of the testsubstances or 0.94 ml of testmedium in case of the blank. The liquid-gas ratio was 2:1 with a total oxygen capacity of ± 28 mg a bottle. The medium was saturated with oxygen at a temperature of 20 °C. The bottles were incubated in the dark at room temperature (18.0-21.4 °C) on a rotary shaker (150 rpm). Every week measurement of the dissolved oxygen was carried out and thereafter samples (2.5 ml) were taken. After centrifugation (5000 x g) the samples were kept frozen at -15 °C until analysis. The volume was adjusted with distilled water (2.5 ml) or in case of a repetitive addition with stocksolution (0.94 ml) and distilled water (1.56 ml). The time schedule of the repetitive additions is given in table 3. The testduration has been 11 weeks. After the test had

been ended, the pH of the solution was measured to check for acidification of the medium, causing an inhibition of the degradation.

2.4 Analytical methods.

2.4.1 Analysis of dissolved oxygen.

The measurement of dissolved oxygen was carried out with the Oxygen 2603 Indicator (Orbisphere Laboratories) at a temperature of 20.0 $\pm$ 0.2 ^{o}C. The precision of the measurement was 0.05 g m^{-3}. The bottles to be measured were put in a waterbath to equilibrate to a temperature of 20.0 ^{o}C and were shaken to reach the equilibrium between oxygen in the gasphase and the measured solution.

2.4.2 Dissolved Organic Carbon (DOC) Analysis.

Dissolved organic carbon was analysed with an Auto-Analyzer method (Technicon), based on an UV destruction in the presence of an oxidative reagent ($K_2S_2O_8$), after which the produced carbondioxide is measured by the decolorization of an indicator solution (phenolphtaleïn) (Schreurs 1978). The precision of the analysis is 0.2 g m^{-3} at concentrations of 25 g m^{-3}. Pretreatment of the samples consisted only out of a centrifugation (10000 x g) step.

2.4.3 Specific analysis.

Only for 4-nitrophenol a specific analysis was available to follow the primary degradation. The analysis of 4-nitrophenol was carried out based on the absorption of 4-nitrophenol at $\pm$ 400 nm in an alkaline solution (Berg, van den 1980). The precision of this analysis is 0.02 g m^{-3} at concentrations of 10 g m^{-3}.

2.5 Calculations.

In the shake flask die-away test the degradation percentages were derived from the measured initial DOC concentration. For the RDA test the degradation percentages were all based on theoretical concentrations, because no samples were taken immediately after the repetitive additions. The theoretical DOC concentration in the test was only verified for the initial addition (table 2).
For the calculation of the theoretical oxygen demand ThOD (table 2) an elimination of the nitro-group of 4-nitrophenol to nitrate and of the amino-group of aniline to ammonia has been assumed.

3 RESULTS AND DISCUSSION

Generally the theorically calculated and measured DOC values agreed well. Only in case of aniline the DOC concentrations determined were 60% of the calculated values. Based on the oxygen uptake it has been concluded that still the theoretically added concentration had been right.
Criteria for determining "ready biodegradability" are:
1. 70% degradation on DOC basis or 60% based on oxygen uptake within a testperiod of 28 days and
2. degradation from 5 to 60 or 70% should occur within a time window of 10 days.

Based on the criteria used for the judgement of "ready biodegradability" only aniline (figure 1) and sodiumbenzoate (figure 2) were found "readily biodegradable" in the standard procedure of the shake flask die-away test (tables 4 and 5). No degradation was observed for 4-nitrophenol (figure 4) and penta-erythritol (figure 5). Diethylene glycol (figure 3) was only degraded to a level of 65% and too slow to be "readily biodegradable".
If the addition of nitrogen and phosphorus nutrients was omitted, generally a decreased degradation rate and level were observed together with a delay in the start of the degradation (table 4). Under these circumstances the degradation for sodiumbenzoate (figure 2) was too slow for "ready biodegradable" and only aniline (figure 1) would have passed the test. In the marine environment N and P limitations occur but the concentration of the compounds will be much lower. It remains doubtful which results should be used for prediction of the fate of organic compounds in the marine environment.

The comparison between the shake flask die-away test and the RDA test shows that no differences occur with regard to the results. Generally the testresults based on dissolved organic carbon degradation corresponded with those based on oxygen uptake (table 4). A part of the carbon taken up is used for growth and not for oxidation. Therefore, as might be expected the degradation levels based on organic carbon elimination are higher than those based on oxygen uptake (table 4).
The results of the RDA testserie with delayed repetitive additions agreed well with the results of the shake flask die-away test. For this testserie aniline (figure 1) and sodiumbenzoate (figure 2) were also found to be "readily biodegradable" (table 5). The degradation of diethylene glycol (figure 3) was too slow and no degradation of 4-nitrophenol (figure 4) and penta-erythritol (figure 5) was determined. The results after 1 week of the RDA test with the normal addition procedure corresponded to that with delayed additions.
Repetitive additions have been applied for the degradable aniline, sodiumbenzoate and diethylene glycol. In case of aniline (figure 1) the repetitive additions had no effect on the degradation levels which remained at 60% (ThOD) and 95% (DOC). The same results were obtained for the 28 days delayed additions.
For sodiumbenzoate (figure 2) the 28 days delayed additions gave also the same results as for the immediately started additions. However, if repetitive additions were carried out the degradation level decreased from 60 to 45% (ThOD) and from 90-95% to 55-65% (DOC).
For diethylene glycol (figure 3) the repetitive additions do

also have a level decreasing effect on the degradation. It became obvious that premature repetitive additions might decrease the degradation to a non-significant level. It is also possible that these premature repetitive additions may accumulate to (semi) toxic levels, resulting in further depression of the biodegradability.
The only testchemical for which the triplicates based on oxygen uptake did not correspond was 4-nitrophenol (figure 4). For some of the bottles a considerable oxygen uptake was determined, but this could not be confirmed by the DOC analysis nor by the specific analysis (figure 6), nor by degradation after a repetitive addition at day 70 (figure 4).
This shows obviously that a significant oxygen uptake is necessary for a reasonable judgement of the degradation. Therefore it is necessary to adapt the liquid-gas ratio in the testbottle to the testconcentration, which is determined by the solubility and the toxicity of the testchemical.
In comparison with the freshwater tests only the results for 4-nitrophenol and penta-erythritol are different. In fresh water tests 4-nitrophenol was "readily biodegradable" in case of optimal testperformance (Berg, van den and Blok 1985, Nyholm e.a. 1984). Both in marine chemostat research (Berg, van den 1985), as in marine field experiments (Kuiper 1982) no degradation, of 4-nitrophenol could be determined.
For penta-erythritol the freshwater results varied, but degradation occurred (Painter and King 1985, Gerike and Fischer 1979).
Measurement of the pH in all bottles of the RDA test (pH 6.8-8.6) showed no pH changes which could have caused the absence of degradation.
The volume adjustments have been carried out with distilled water. Finally this caused a decrease in the salinity with a concomitant increase in the oxygen saturation value from 7.65 to 7.85 g m^{-3}. The final results have not been influenced much because the blank was treated the same way, but in future experiments the volume adjustements should be carried out with sterile testmedium.
The advantages of the RDA test with regard to the shake flask die-away test are the combination of the two methods of measurement, the possible use for testing poorly water soluble and even volatile compounds and the kinetic information obtained from the repetitive additions.
A disadvantage of the RDA test is the more time-consuming procedure, because of the oxygen measurements and the performance of the repetitive additions and aerations. This RDA test took about 100 hours, as the shake flask die-away test has been done in 70 hours.

4 CONCLUSIONS

In these two sea water biodegradability tests studied: the shake flask die-away test and the repetitive die-away test the testsubstances aniline and sodiumbenzoate were found "readily biodegradable". Diethylene glycol also was degraded but did not met the pass criteria. Penta-erythritol and 4-nitrophenol were not degraded in any of the testseries.

Omittance of the nitrogen and phoporus nutrients has a decreasing effect on degradation rate and level and delays the start of the degradation. Research is needed to determine what role N and P limitations plays in determining the biodegradation capacity.

The gas-liquid ratio in the RDA test should be chosen according to the applied testconcentration, which is determined by solubility and especially toxicity of the testsubstance.

It is recommended to start (and continue) the repetitive additions only if the degradation has reached a certain level (e.g. >50%) or rate (e.g. >30% in 1 week). Premature repetitive additions can give rise to a decreased and non-significant degradation level and possibly toxic effects.

The repetition of the additions in the RDA test makes it possible to distinguish between testsubstances which show the same behaviour in a single addition test.

The results of the shake flask die-away test and the repetitive die-away test agreed well.
In comparison with the shake flask die-away test the RDA test is preferred because of its many advantages, in spite of its more time-comsuming design.

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TABLE 1. Characteristics of the collected sea water.

Date of collection	:	1.5.85
Sample location	:	52o36' N, 4o24' E
Temperature at the time of collection	:	7.4 ^{o}C
Salinity	:	32.5 g kg^{-1}
Concentration dissolved organic carbon	:	4.5 g m^{-3}
Temperature during transport	:	15 ^{o}C
Pretreatment of the seawater	:	filtration through a paper filter Ederol no. 1, 0 18.5 cm, 0.11 mg
DOC concentration after filtration	:	2.4 g m^{-3}

Microbial colony count, before treatment and just before start of the test:

Before treatment	:	>> 1.000 and < 1.000.000 ml^{-1}
Before start of the test	:	>> 1.000 and < 1.000.000 ml^{-1}

- the number of colonies could not be determined accurately because of too high dilution of the seawater, the lower limit has been established with seawater plated on 3.5.85 -

TABLE 2. The theoretical and measured concentrations of the stocksolutions and the additions of the testsubstances.

	concentration in the stocksolution ($g\ m^{-3}$)			initial concentrations ($g\ m^{-3}$)			
	theoretical		measured	theoretical			measured
testsubstance	substance	DOC	DOC	substance	ThOD	DOC	DOC
aniline	5030	3894	2130	4.73	11.39	19.6	8.6
sodiumbenzoate	7000	4083	3775	6.58	10.97	20.5	18.5
diethylene glycol	9124	4132	4240	8.58	12.95	20.8	20.1
4-nitrophenol	4015	2080	2400	3.77	6.08	10.5	8.3-9.6
penta-erythritol	9001	3971	4040	8.46	11.94	20.0	19.9

TABLE 3. Time schedule of the repetive additions in the RDA test.

		Additions after ... weeks.										
	testsubstances	0	1	2	3	4	5	6	7	8	9	10
standard procedure	aniline	x	x	x	x							
	sodium benzoate	x	x	x	x							
	diethylene glycol	x			x	x	x					
	4-nitrophenol	x										x
	penta-erythritol	x										
delayed repetitive additions	aniline	x				x	x	x				
	sodiumbenzoate	x				x	x	x				
	diethylene glycol	x								x		
	4-nitrophenol	x										x
	penta-erythritol	x										

TABLE 4. Degradation percentages of the testsubstances in the various tests based on oxygen and dissolved organic carbon.

test	shake flask die-away test				repetitive die-away (RDA) test					
degradation parameter	DOC				DOC	ThOD	DOC	ThOD	DOC	ThOD
after x days	28	77	28	77	28		77		77	
remarks	with N P nutr.		without NP nutr.		delayed repetitive				standard procedure	
testsubstances										
aniline	90	90	74	79	94	104	97	92	96	83
sodiumbenzoate	96	97	49	92	98	98	62	46	71	47
diethylene glycol	28	65	20	52	33	27	49	28	33	19
4-nitrophenol	-3	-6	-11	1	11	16	14	23	15	10
penta-erythritol	-4	-9	-9	-16	0	5	8	8	7	2

TABLE 5. Degradation results for the testsubstances interpreted to "ready biodegradability".

+ = "readily biodegradable" ± = biodegradable - = non biodegradable

test	shake flask die-away test		repetitive die-away (RDA) test			
remarks	with nutrients	without nutrients	non-repetitive		standard procedure	
degradation parameter	DOC	DOC	DOC	ThOD	DOC	ThOD
testsubstances						
aniline	+	+	+	+	+	±
sodiumbenzoate	+	±	+	+	+	±
diethylene glycol	±	±	±	±	±	±
4-nitrophenol	-	-	-	-	-	-
penta-erythritol	-	-	-	-	-	-

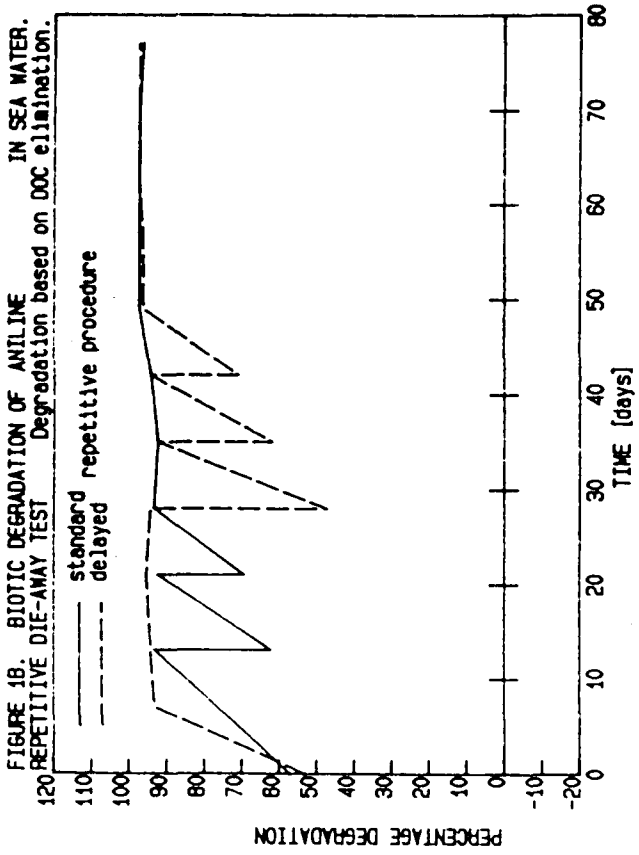


FIGURE 1B. BIOTIC DEGRADATION OF ANILINE IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on DOC elimination.
standard repetitive procedure
delayed
PERCENTAGE DEGRADATION
TIME [days]

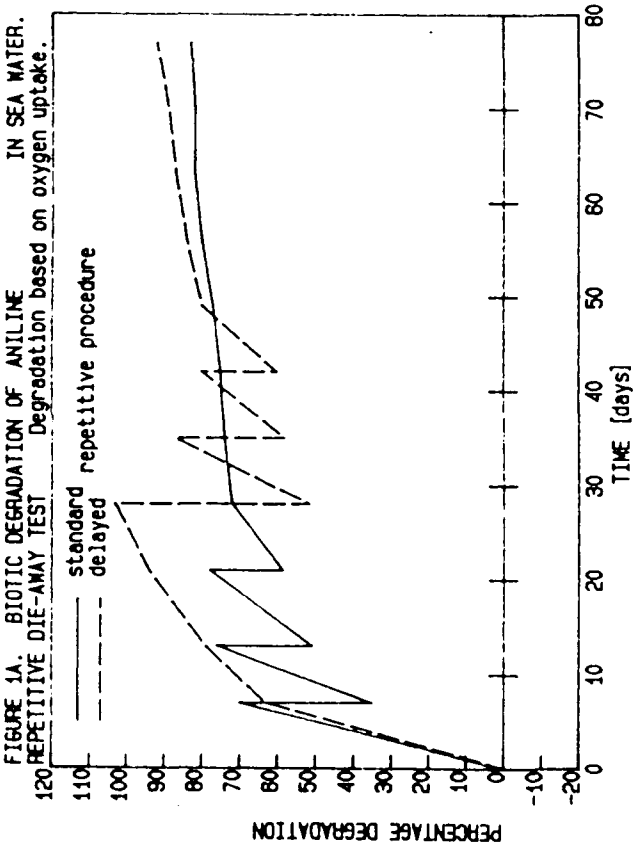


FIGURE 1A. BIOTIC DEGRADATION OF ANILINE IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on oxygen uptake.
standard repetitive procedure
delayed
PERCENTAGE DEGRADATION
TIME [days]

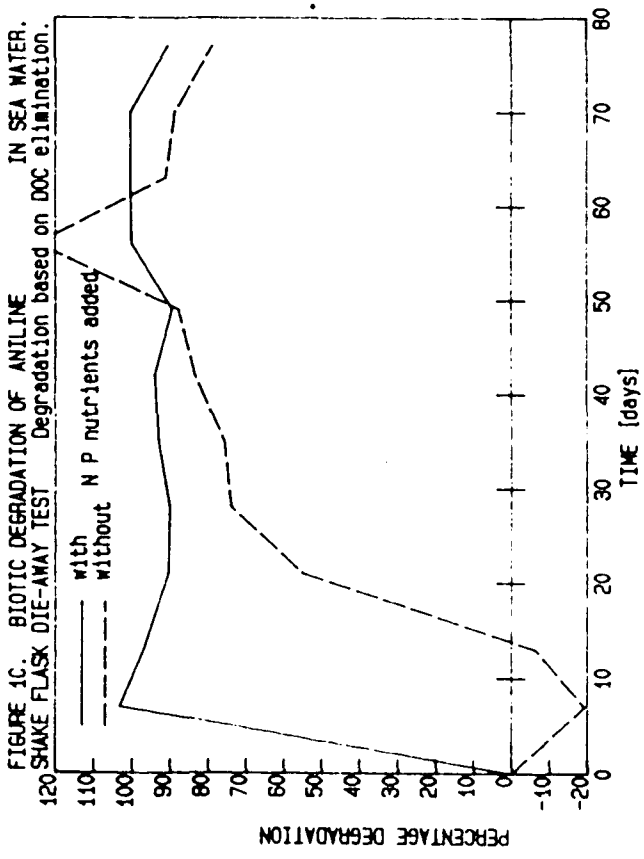


FIGURE 1C. BIOTIC DEGRADATION OF ANILINE IN SEA WATER.
SHAKE FLASK DIE-AWAY TEST Degradation based on DOC elimination.
with
without N P nutrients added
PERCENTAGE DEGRADATION
TIME [days]

FIGURE 2A. BIOTIC DEGRADATION OF SODIUM BENZOATE IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on oxygen uptake.

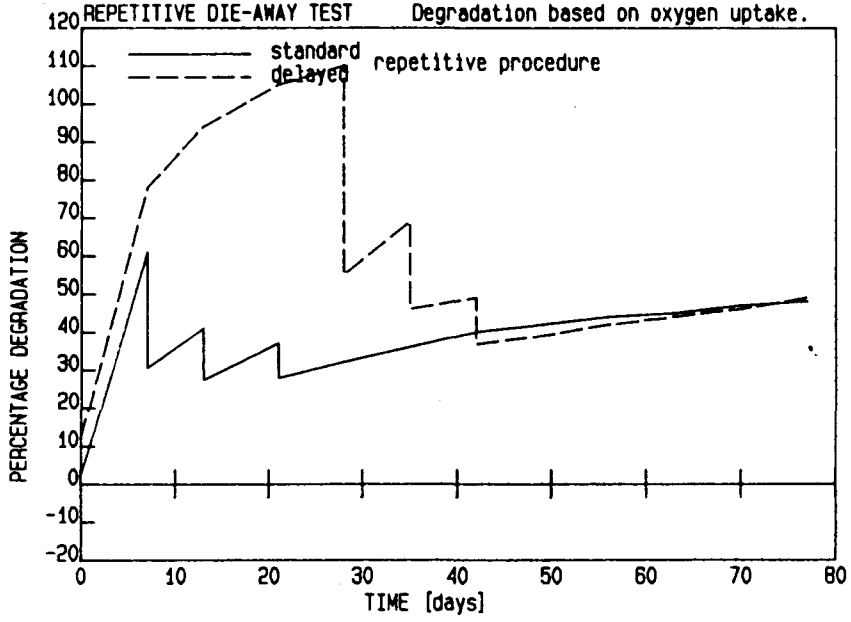


FIGURE 2B. BIOTIC DEGRADATION OF SODIUM BENZOATE IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on DOC elimination.

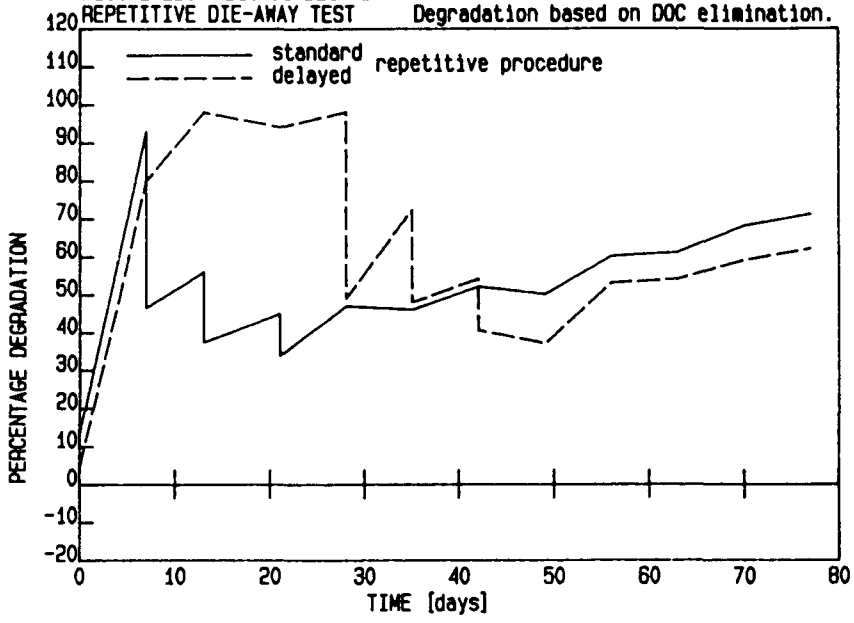
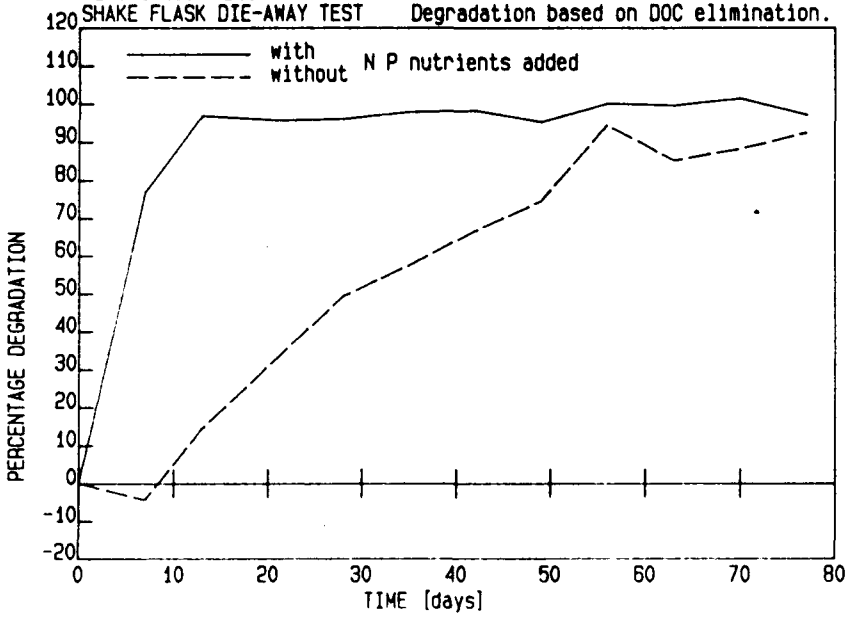


FIGURE 2C. BIOTIC DEGRADATION OF SODIUM BENZOATE IN SEA WATER.
SHAKE FLASK DIE-AWAY TEST Degradation based on DOC elimination.



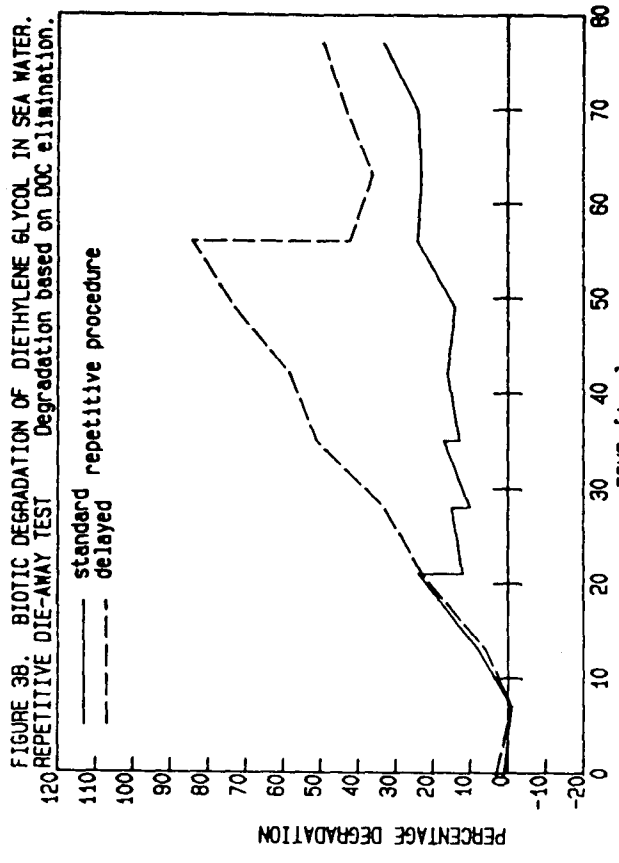


FIGURE 3B. BIOTIC DEGRADATION OF DIETHYLENE GLYCOL IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on DOC elimination.
standard repetitive procedure
delayed
PERCENTAGE DEGRADATION
TIME [days]

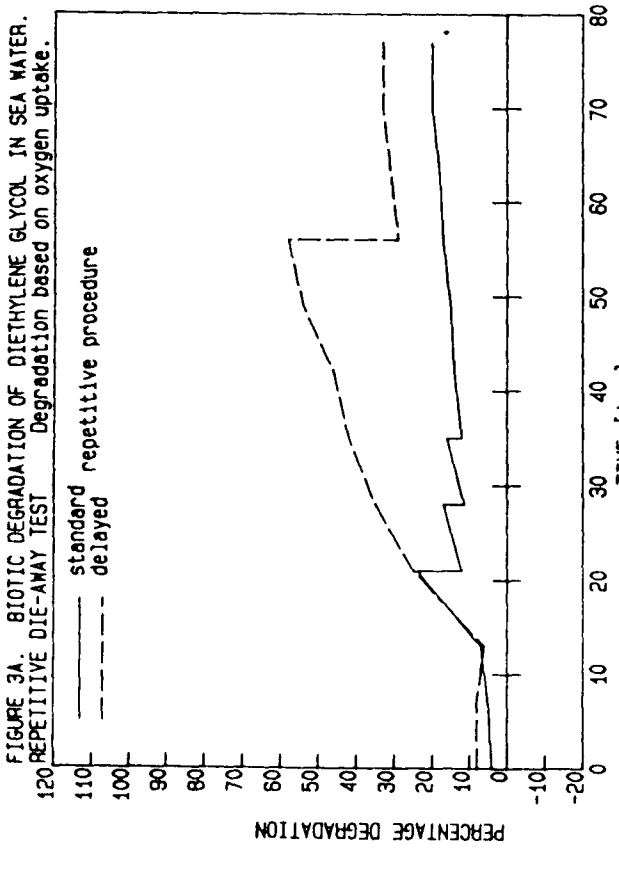


FIGURE 3A. BIOTIC DEGRADATION OF DIETHYLENE GLYCOL IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on oxygen uptake.
standard repetitive procedure
delayed
PERCENTAGE DEGRADATION
TIME [days]

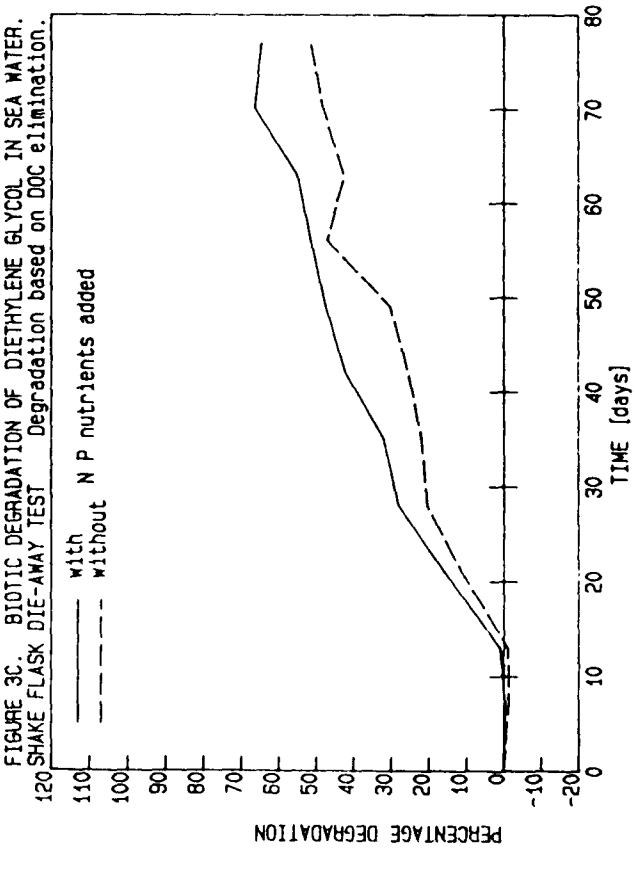


FIGURE 3C. BIOTIC DEGRADATION OF DIETHYLENE GLYCOL IN SEA WATER.
SHAKE FLASK DIE-AWAY TEST Degradation based on DOC elimination.
with N P nutrients added
without
PERCENTAGE DEGRADATION
TIME [days]

FIGURE 4A. BIOTIC DEGRADATION OF 4-NITROPHENOL IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on oxygen uptake.

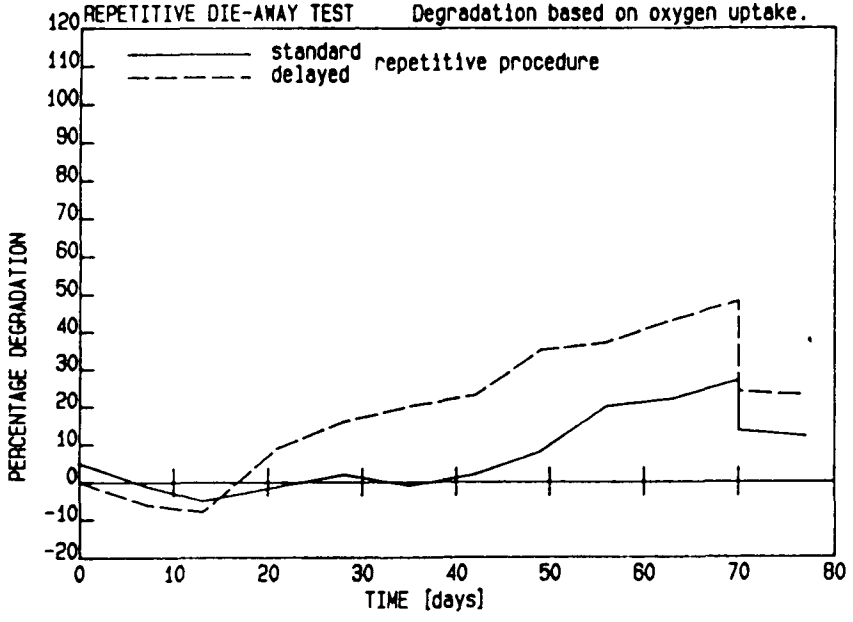


FIGURE 4B. BIOTIC DEGRADATION OF 4-NITROPHENOL IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on DOC elimination.

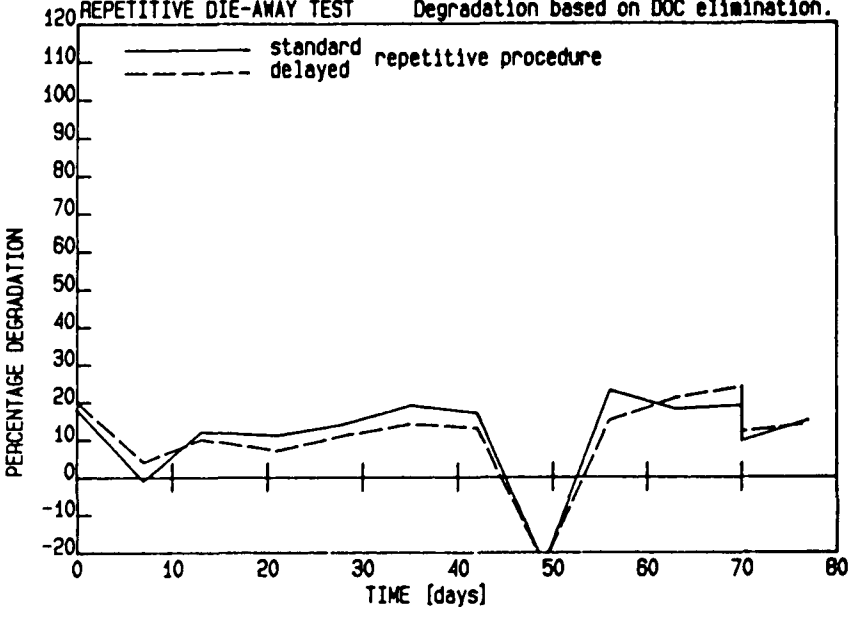


FIGURE 4C. BIOTIC DEGRADATION OF 4-NITROPHENOL IN SEA WATER.
SHAKE FLASK DIE-AWAY TEST Degradation based on DOC elimination.

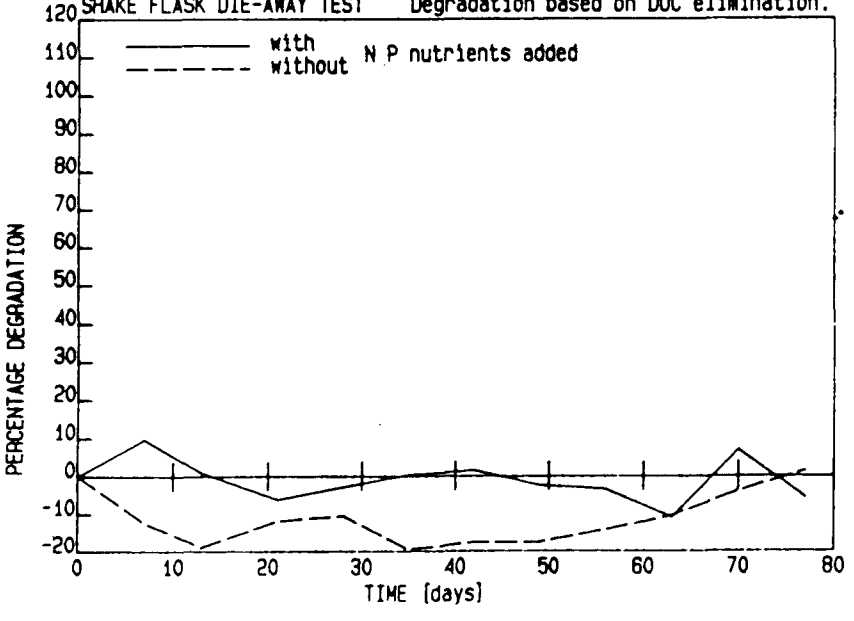


FIGURE 5A. BIOTIC DEGRADATION OF PENTA-ERYTHRITOL IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on oxygen uptake.

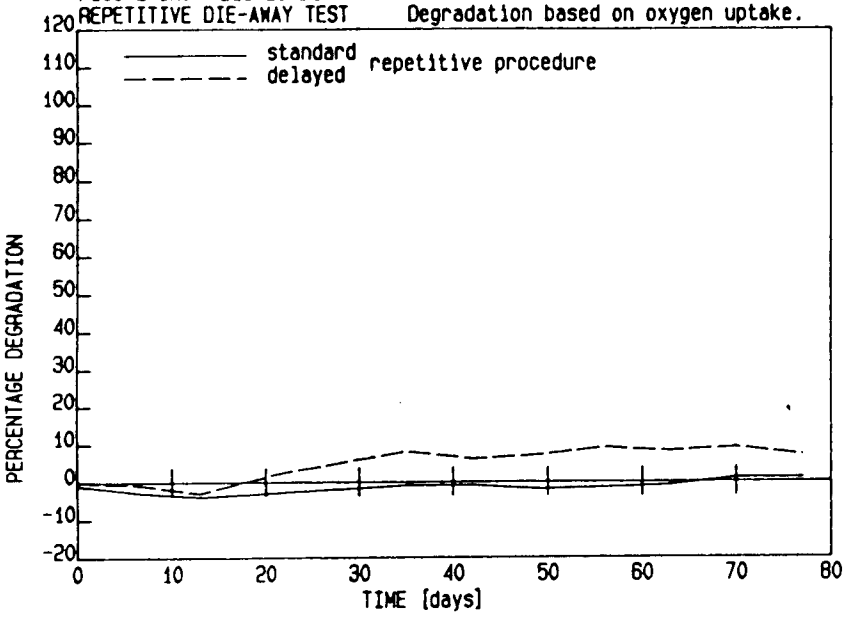


FIGURE 5B. BIOTIC DEGRADATION OF PENTA-ERYTHRITOL IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on DOC elimination.

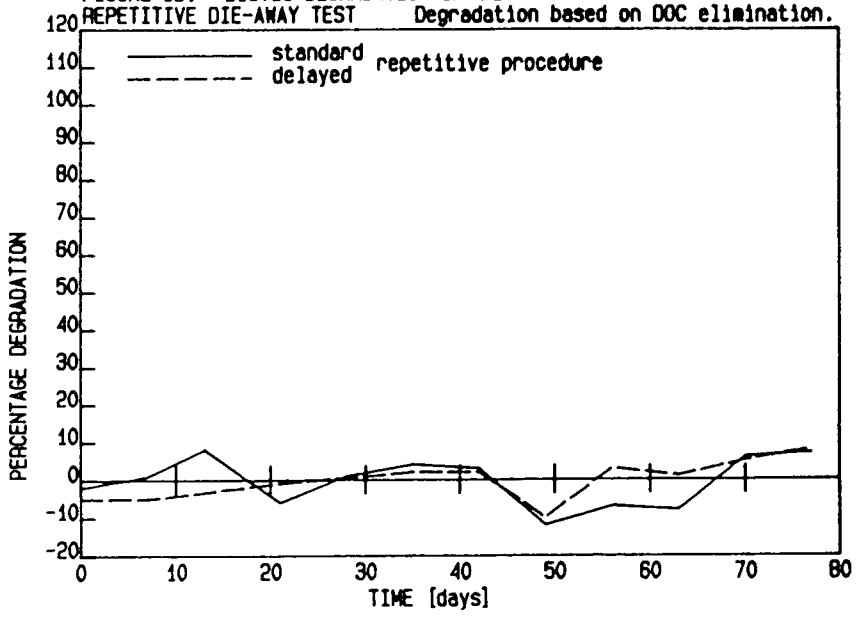
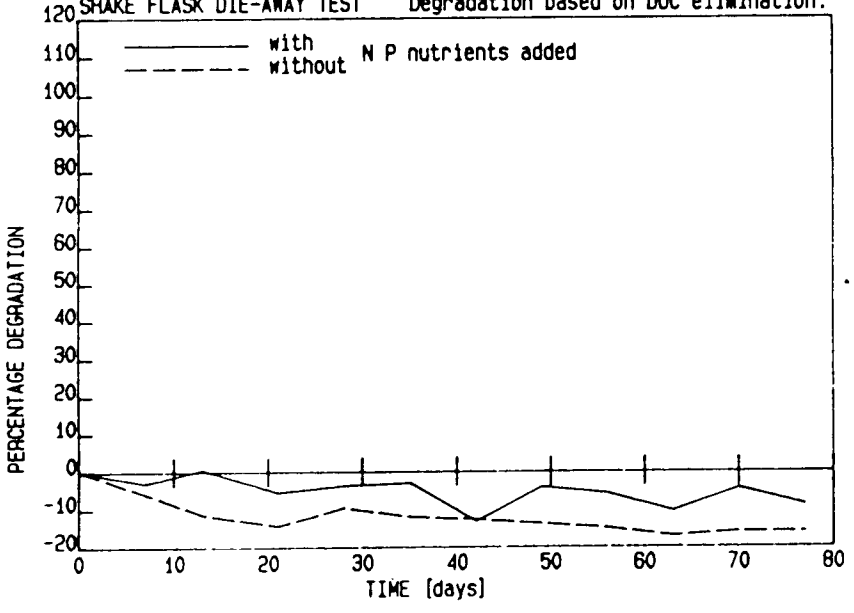


FIGURE 5C. BIOTIC DEGRADATION OF PENTA-ERYTHRITOL IN SEA WATER.
SHAKE FLASK DIE-AWAY TEST Degradation based on DOC elimination.



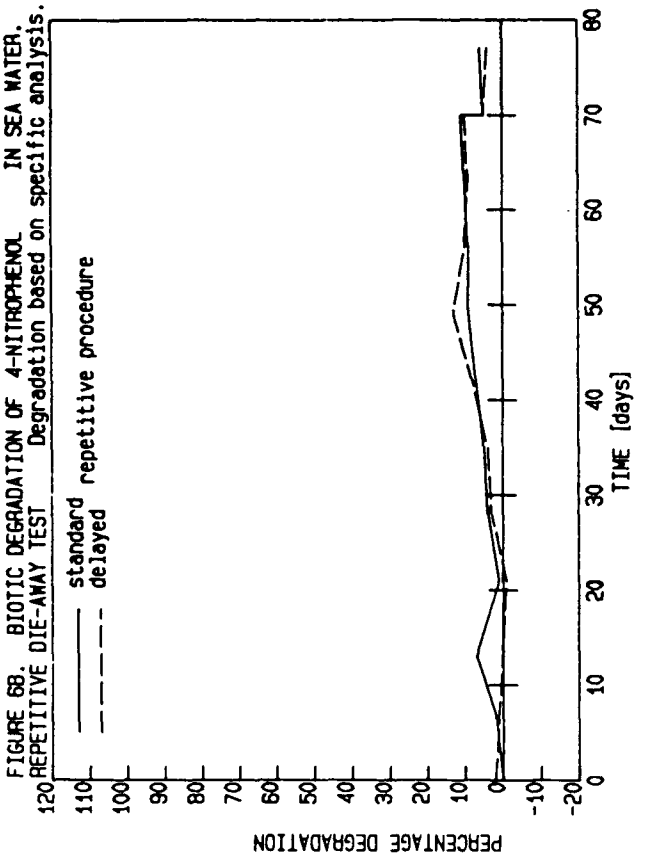


FIGURE 6B. BIOTIC DEGRADATION OF 4-NITROPHENOL IN SEA WATER.
REPETITIVE DIE-AWAY TEST Degradation based on specific analysis.
standard repetitive procedure
delayed
PERCENTAGE DEGRADATION
TIME [days]

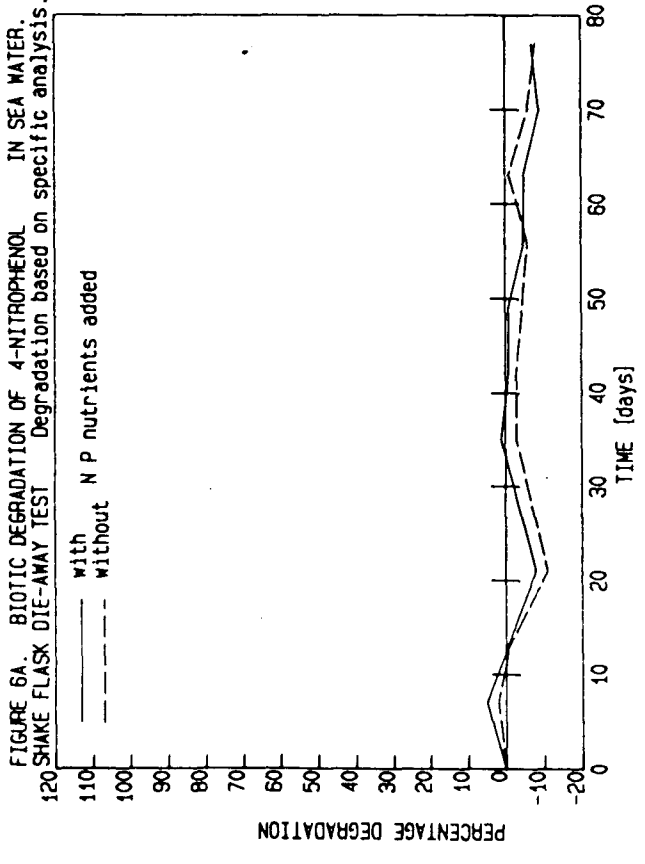


FIGURE 6A. BIOTIC DEGRADATION OF 4-NITROPHENOL IN SEA WATER.
SHAKE FLASK DIE-AWAY TEST Degradation based on specific analysis.
with
without N P nutrients added
PERCENTAGE DEGRADATION
TIME [days]