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ABSTRACT

The degradation of Aroclors 1242 and 1016 has been studied using two biphenyl degrading organisms C_2 and C_3 for periods up to 100 days. Results indicate good biodegradability for all components of Aroclor 1016.

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SUMMARY OF RESULTS

Note!

1. In the presence of biphenyl, Aroclors 1242 and 1016 are degraded by both C2 and C3 organisms. After 100 days Aroclor 1016 residues are less than 5% the original concentration with either organism. Aroclor 1242 is degraded to a lesser extent, there being approximately 5 - 10% remaining with the C2 organism and 15 - 20% with the C3 organism after 100 days.

2. The longest half life for any component of Aroclor 1016 is approximately 25 - 30 days.

3. For pure compounds, there is evidence to suggest that C2 and C3 have different rates of degradation and each is specific to certain basic structures; in complex mixtures this does not appear to be the case since C2 and C3 degrade the components of Aroclor 1016 at approximately the same rate and to approximately the same amount.

4. Analytical developments show that the electron capture detector response to individual components can be predicted with an accuracy sufficient for most environmental analytical problems using packed columns and that with capillary columns quantitative measurements can be made upon degraded P.C.Bs at the 0.01 ppm level

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INTRODUCTION

A previous review report (No. MP 10103) discussed results obtained for the biodegradation of pure chlorinated biphenyls using a biphenyl degrading organism C2 (the Sudan organism) obtained from Professor Charles Evans of the University College of North Wales, Bangor. The effect of C2 on commercial and development samples (1242, 1016, 717, 762 etc.) was also discussed but, because of analytical difficulties encountered in the identification of specific components in the mixtures, only very limited interpretation could be made of the results.

Since the issue of that report the identification of components has been greatly improved and, in the work reported here, Aroclors 1242 and 1016 have been analysed after submission to biodegradation in the presence of biphenyl by two cultures C2 and C3 for one hundred days. Improvements have been made in the quantitative analysis and it has been possible to study the change in individual isomers at levels below 0.05 parts per million.

1. DETAILS

1.1 Organisms used for degradation of Aroclors 1242 and 1242B

Two organisms both received from Professor Charles Evans at University College of North Wales, Bangor have been used for biodegradation.

The first organism C2 is that originally received and used for the initial work on the degradation of pure compounds. This was isolated in a soil sample from the Sudan and is reported as being a nocardium of the order actinomycetes.

The second organism C3 was isolated from a canal mud sample in the screening work carried out by Professor Evans student, C. Harrison. This is reported as being of the pseudomonas family.

The mode of attack by these organisms on biphenyl has been studied by Professor Evans' group and a simplified scheme is given later in the Appendix.

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1.2. Preparation and maintenance of stock cultures of biphenyl degrading organisms

The procedure used is identical to that reported earlier (Review Report No. MP 10103).

1.3. Procedure for studying P.C.B. biodegradation

A number of changes have been made in the procedure used to study biodegradation. These have been made to increase the number of samples examined at any time and to improve the qualitative and quantitative accuracy of the analytical method.

(i) Addition of Orbital Incubator

To increase capacity, an orbital incubator was purchased (Gallenkamp) with a capacity of 15 x 1000 ml, 28 x 500 ml. or 36 x 250 ml. flasks.

Past experiments have been designed so that the complete contents of any shake flask have been extracted and analysed. A more attractive method would be the use of aliquots whereby the fate of a sample in any flask could be studied over a period. This method was rejected earlier because (a) the flask shaker was too small to handle the larger volumes needed for aliquot work and (b) it was strongly suspected that the sample would not be homogeneously dispersed in the medium but may be partly adsorbed on the flask wall.

In view of the increased size of flask, which could be accommodated in the incubator, it was felt that the use of aliquots should be evaluated - if successful the variation in results caused by the use of separate flasks would be eliminated and a more varied biodegradation programme could be designed.

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The evaluation was carried out as follows :

Six 1 litre flasks were dosed with Aroclor 1242 in the normal way. Each flask contained 700 mls of water with an Aroclor 1242 concentration in the flask equivalent to 5 ppm. If the aliquot method was suitable each 100 ml aliquot of water should contain 5 ppm of 1242.

The flasks were shaken in the incubator (1.25 r.p.m., 25°C) for eight days after which a 100 ml aliquot was taken from each flask. Of the remaining 600 mls of water in each flask, 500 ml was discarded and the remaining 100 ml recovered for analysis. These first and final aliquots were extracted with pentane; in the case of the final 100 ml aliquot the pentane was first used to remove any Aroclor 1242 adhering to the flask. The pentane extracts were analysed using - packed column gas chromatography. Results are given below, the figures in brackets are the theoretical figures which should be obtained if the aliquot system is reliable.

	1242 content (ppm) 1st Extract			1242 content (ppm) Final Ext
1	1.4	(5.0)	×6 = 8.4	24.4 (5.0)
2	1.7	"	10.2	22.6 "
3	0.8	"	4.8	24.5 "
4	0.7	"	4.2	35.7 "
5	1.3	"	7.8	25.5 "
6	1.0	"	6.0	29.8 "

It can be seen from these results that the aliquot system is not suitable for aqueous samples containing Aroclors and that adsorption to the container wall is so large that this factor is likely to be important in the interpretation of any data from similar experiments.

Depletion data from this experiment may have a "wall effect".

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Thus the problem still remained of how to obtain the maximum information using a limited number of samples. In previous work each sample under study had three flasks prepared as follows :

1. Sample + Culture + Nutrients + Biphenyl
2. " " " no Biphenyl
3. " " " " " + $HgCl_2$

In every case to date, the sample with mercuric chloride has given results in agreement with a normal standard not submitted to biodegradation and therefore this series of flasks has been excluded from the present work. This has increased the relative capacity of the incubator by 30%.

1.4. Improvements in the Analytical Procedure

The details of the analytical method used are given in the Appendix.

1.4.1. Identification of Components

The development of a suitable analytical method has been a major problem in the study of the biodegradation of complex commercial and development samples of P.C.B's. Packed column gas chromatography using the electron capture detector will give a measure of whether a complex sample is undergoing biodegradation but the resolution attainable is not sufficient to determine the fate of individual components. Without this knowledge and because the response of the E.C. detector to individual P.C.Bs varies with structure, quantitative results must be viewed with caution.

It is therefore necessary to use high resolution capillary columns to obtain detailed information but a further complication is introduced in that there are insufficient pure P.C.Bs available to identify all the peaks in a chromatogram (up to 50 in 1242) using the normal procedure of "spiking".

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In the early stages of this work it was therefore decided to concentrate upon the use of capillary columns coated with the non polar phase Apiezon L. While these have a poorer resolving power than many polar columns of equivalent length they have the major advantage in eluting components with respect to boiling point and this property can often be predicted from a knowledge of structure and the behaviour of similar compounds.

Emperical rules were drawn up to predict the probable retention times of unavailable P.C.Bs, the rules being based upon steric factors, molecular weight etc. Such rules aided in the identification of certain components but were not sufficiently detailed or established to cover all components.

This approach was discussed with Welti, Sissons and Telling at Unilever who were working along similar lines. A thorough investigation by this group using confirmatory techniques of N.M.R., mass spectrometry and the spiking procedure resulted in complete (within experimental error) identification of all components in 1242, 1254 and 1260.

This work is awaiting publication but we have had access to the data for the previous six months and, combined with our own, this has been used for the identification of all components named in the results section.

1.4.2. Quantitative Accuracy

While improvements have been made in identification of components, the quantitative accuracy of the gas chromatographic method has still to be improved. Use of capillary columns mainly overcomes the difficulty of merged peaks but it has a disadvantage in that it requires more concentrated samples than packed column gas chromatography.

To overcome this, the concentration proceudre described in the Appendix was developed. This involves concentration of the pentane extract to a low volume followed by addition of a small known amount of decane. This allows complete removal of the pentane giving a concentration factor of up to 200X without significant loss of P.C.B. components and enables accurate measurements to be made on degraded components at levels as low as 0.05 ppm.

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1.4.3. Calibration

Work by Emery et al (M.C. Physical Chemistry Method 70-20. N.N. 1348322) has shown that the F.I.D. response factors of chlorinated biphenyls depend upon chlorine content and that within any group of compounds of the same chlorine number, structural differences have a minimal effect upon response factor. Thus, in any high resolution chromatogram, if the chlorine numbers of the peaks are known, response factor correction can be applied and a true figure for either the composition of the Aroclor or its concentration in a sample can be calculated.

Unfortunately, because of sensitivity limitations, the F.I. detector cannot be used for determinations involving very low levels of P.C.Bs and the electron capture detector must be employed.

There has been much speculation on the quantitative accuracy of this detector for P.C.B. analysis since it is known from other applications that its response is very dependent upon structure; work with pure components within Monsanto and elsewhere (eg. see Zitko et al Bull Environ Contam and Toxicol 1971 6 2 pp 160-163) has indicated that with P.C.Bs there can be large differences in response for compounds of the same chlorine number but different structure

$$\text{e.g.} \quad \frac{\text{Response 2 4}' \text{ dichloro biphenyl}}{\text{" 44}' \text{ " "}} \approx \frac{12}{1}$$

It was therefore decided that for accurate quantitative analysis using the E.C. detector the most reliable approach would be to determine the ratio for each component of the E.C.D. to F.I.D. response, use this to convert the E.C.D. chromatogram to an "equivalent" F.I.D. chromatogram and apply the F.I.D. correction factors calculated by Emery et al.

The ratio of $\frac{\text{E.C.D.}}{\text{F.I.D.}}$ response is relatively easily obtained by running the same standard sample on a capillary column using a flame ionisation detector, then repeating the experiment (after dilution) with an electron capture detector (See Figs 9 & 10). Peak areas are calculated then ratioed. The ratios obtained for each component can then be used for all subsequent samples provided that the column used has suitably high resolution.

Not correct figure
Fig 9 is metabolic pathway not a gas chromatogram
Should be Fig 10 + 11.

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Figs 10+11 are not very similar to one

In carrying out this experiment and also in previous work it was noted that for all the concern about the unpredictable response of the E.C.D. the chromatograms obtained with the two detectors were remarkably similar and calculation of the $\frac{\text{E.C.D.}}{\text{F.I.D.}}$ ratios for all peaks shows that large differences between the two detector responses are only observed for a small number of components.

It is suspected that the similarity of E.C.D. response factors of compounds of the same chlorine number but different structure is related to the fact that almost all the components of Aroclor 1242 (and higher Aroclors) contain at least one chlorine in the ortho position. Such compounds will have non coplanar benzene rings, there will be much reduced conjugation and this general property may have such a large effect upon E.C.D. response as to make other structural differences of much lower importance for components of the same chlorine number. This would explain the anomalous E.C.D. response factor of the unhindered 44' dichloro biphenyl.

Initial measurements with pure 33' dichloro biphenyl show that this compound also has a low E.C. response almost equivalent to that of the 44' dichloro biphenyl.

As discussed, if high resolution capillary columns are used the $\frac{\text{E.C.D.}}{\text{F.I.D.}}$ ratios coupled with the F.I.D. will give an accurate quantitative figure for P.C.B. content at low ppm and ppb levels.

If the $\frac{\text{E.C.D.}}{\text{F.I.D.}}$ ratios for each set of components with the same chlorine number are averaged (neglecting components of very low concentration) an unexpected result is obtained viz.

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To convert dichlorobiphenyl E.C.D. areas to F.I.D. areas using $\frac{E.C.D.}{F.I.D.}$ ratio divide by C

" " tri " " "

II. RESULTS

The results obtained for Aroclor 1242 and 1016 together with interpretation and discussion are given in Sections 11.3 and 11.4.

II.1. Sources of Error in Interpretation

In interpreting the results it must be emphasised that there are still unknown factors which may cause significant errors. Two such sources of error are considered below :

A. Identification of components

As discussed earlier components have been identified using one or a combination of the techniques of spiking, N.M.R. and mass spectrometry. All such techniques are open to error in a complex sample of this type, even, in some cases, the authenticity of the original component used for spiking may not be guaranteed.

B. The graphs for degradation of components are prepared from results obtained for separate samples of Aroclors taken at 52 and 100 days. Thus while the samples have been prepared under as identical conditions as possible, once biodegradation is initiated each sample is an isolated system and different rates and/or processes may occur. Every attempt has been made to ensure reproducible conditions but it has been found in a number of previous runs that occasionally a 50 day sample shows better degradation than a 100 day sample.

An attempt to reduce the chances of this situation occurring will be made in future by the introduction of the "viable cell count". This is discussed below.

II.2 Viable Cell Count

In the discussion on degradation of 1242 and 1016 the results have been interpreted without considering in detail the effect of time on the growth of the organisms in samples in a closed system.

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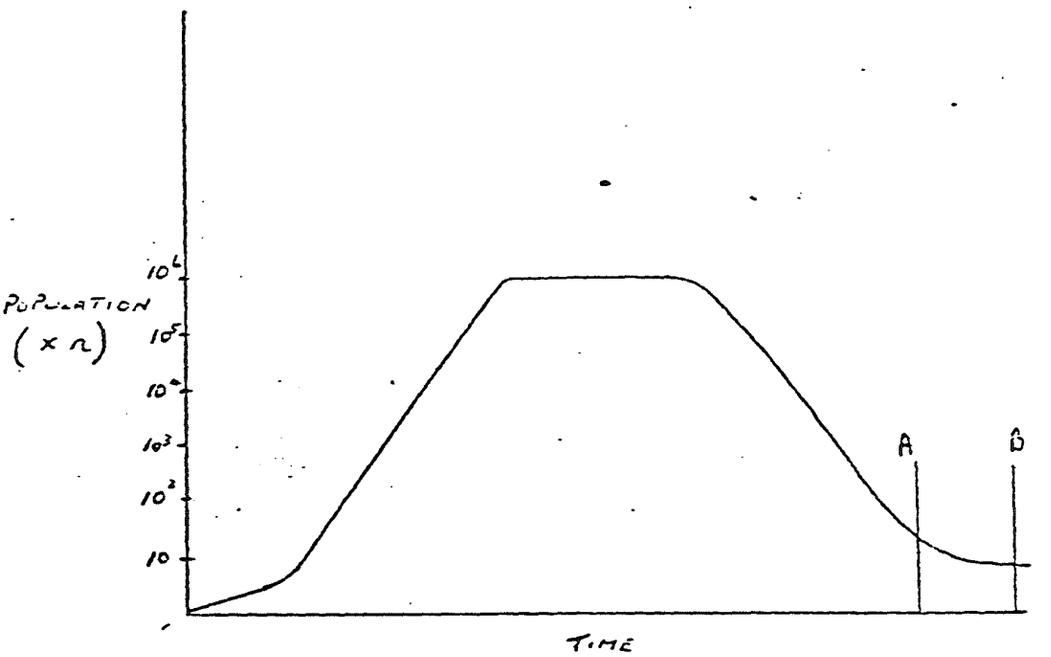
It is known that under optimum conditions a culture of micro organisms will have a growth curve for viable cells similar to that drawn below :



In the experiments described, the viable cell population has not been counted when samples have been taken, as it has been assumed that where the rate of degradation is considerable, i.e. during the first 50 days, this was unnecessary. However, when the residual material becomes very low i.e. after 100 days, the viable cell population may be in the region AB on the graph. Here the reduction in cell count is several orders of magnitude below the maximum count but the concentration of P.C.B. is reduced by less than two orders of magnitude. Thus the relative efficiency of degradation in the closed system will reduce considerably with time.

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This is not the case in most normal environments where the P.C.B. could be exposed to a number of organisms at different times, each working at high efficiency.

with O_2 added

11.3 Biodegradation of Aroclor 1016

Fig 7 gives only 17 components

The percentage degradation for Aroclor 1016 by C2 and C3 after 52 and 100 days is given in Table I and schematic diagrams of the rates of degradation of the individual components are given in Fig. 7.

From experiments following individual components of 1016

The rate graphs are meant for illustration only since insufficient points for each component have been obtained to allow accurate decay curves to be obtained. If the graphs are used as drawn, to obtain an approximate half life for each component (i.e. time to 50% degradation) then it can be estimated that the slowest degrading component has a half life of 33 days with C3 and 28 days with C2. If however, degradation is assumed to be exponential (which is inferred if component half life is used as a measure of efficiency of degradation) the figures are lower still probably in the region of 10 - 20 days. In practise even these numbers are on the high side since, as has been discussed earlier, efficiency of biodegradation will fall off with time in a closed system.

The graphs show that degradation appears to be smooth and relatively rapid with both organisms. Small amounts of degradation products can be seen in the early part of the chromatogram but the retention times of these confirm that they are not chlorinated biphenyls.

It has been noted earlier that the mechanisms of biodegradation for C2 and C3 with biphenyl differ (see Appendix Fig. 9) and it would therefore be expected that the high resolution gas chromatograms of Aroclor 1016 degraded by the two organisms would show major differences. In practice while there are differences these are relatively small (in comparing chromatograms it should be borne in mind that although a 1 ppm. standard is used the original concentration was 5 ppm.)

This suggests that when the organisms after adaption with biphenyl are exposed to a wide range of components they are capable of further adaptability to suit the available food source, that degradation is uniform and extensive after 52 days shows that adaptation is not difficult. This is supported for C2 by the evidence in the literature that the Actinomycetes family are known to adapt to a wide range of food substrates.

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II.4 Biodegradation of Aroclor 1242

The percentage degradation for Aroclor 1242 by C_2 and C_3 after 52 and 100 days is given in Table I and the apparent rates of degradation of the components are given in Fig. 8.

The comments made earlier concerning half lives of the components of Aroclor 1016 also apply in this case.

The graphs and the chromatograms from which they are derived show that biodegradation appears to be more complicated and while degradation is considerable after 100 days the rates of degradation for many components do not follow smooth curves.

Reasons can be given for the differences between the biodegradation of 1242 and Aroclor 1016 but these must be treated with caution : -

Two main differences are apparent (a) certain components in 1242 appear to decrease in the first 52 days then increase in the second period. (b) the rates of degradation for C_2 and C_3 for individual components differ far more than with Aroclor 1016.

Both differences may be caused by the effect of the higher chlorinated components which are present in greater concentration in 1242 than in 1016.

In the first case, these may be degrading to give lower molecular weight components which elute with the same retention times as the lower chlorinated biphenyls or, in fact, the higher chlorinated biphenyls may lose chlorine and gain hydrogen by an unknown process to give the lower chlorinated compounds.

In the second case, initial degradation of the higher chlorinated materials may give rise to products which have different toxicities for C_2 and C_3 and, in a medium of increased toxicity, these organisms will utilise the available food by different mechanisms than they would in a non toxic medium.

Again, in future work, the effect of toxicity may be detected using the "viable cell count" procedure discussed in Section II.2.

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11.5 Relationship between structure and ease of biodegradation

The present results on 1242 and 1016 for C2 and C3 provide an additional means of studying the effect of structure upon biodegradability and it should be possible to obtain a considerable amount of information from relatively few experiments. Because of the complex nature of the degradation process and the apparent ease of adaptation of the organisms it is felt that the present data is insufficient, and that further experiments should be completed before detailed correlation between structure and biodegradation are considered or attempted.

III. FUTURE WORK

III.1. Quantitative Accuracy

It has been suggested earlier (Section 1.4 III) that the response of the electron capture detector is largely conditioned by the non coplanarity of the benzene rings in the chlorinted diphenyl molecule.

Samples of unhindered chlorinated niphenyls have been supplied by Unilever and these will be examined to decide whether this postulate is true.

III.2 Sample Programme

a) A repeat series of Aroclors 1242 and 1016 is underway using C2 and C3 and degradation will be followed until complete disappearance (50 day ready Sept. 6th).

b) A mixed culture of C2 and C3 is being used to attempt biodegradation of Aroclors 1242 and 1016 to determine whether the organisms can co-exist and still work equally effectively.

c) A river water die away test on 1242 and 1016 has been initiated for a long term study.

d) A variety of development samples including chlorinated terphenyls has been subjected to biodegradation by C2 and C3. The results on these will be reported separately when complete.

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III.3 Viable Cell Count

The viable cell count method is being carried out to obtain expertise in this technique. It will then be applied to each sample submitted for analysis.

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Appendix

1. Table 1 % Biodegradation with C_2 and C_3 after 51 and 100 days
2. Table II Half lives of components of Aroclor 1016
3. Fig.1. Chromatogram of 1 ppm of Aroclor 1242 (0 days)
4. Fig.2. " 5 ppm " " " @ 51 days (C_2)
5. Fig.3. " " " " " 100 days (C_2)
6. Fig.2a. " " " " " 51 days (C_3)
7. Fig.3a. " " " " " 100 days (C_3)
8. Fig.4. " 1 ppm " " 1016 0 days
9. Fig.5. " 5 ppm " " " 51 days (C_2)
10. Fig.6. " " " " " 100 days (C_2)
11. Fig.5a. " " " " " 51 days (C_3)
12. Fig.6a. " " " " " 100 days (C_3)
13. Fig.7. Rates of degradation for components of Aroclor 1016 (C_2 and C_3)
14. Fig.8. " " " " " " " 1242 (C_2 and C_3)
15. Method for G.C. Analysis of degraded samples
16. Fig.9. Metabolic Pathways for degradation of biphenyl with C_2 & C_3
17. Fig.10. Chromatogram of Aroclor 1242 F.I. detector.
18. Fig.11. " " " " E.C. detector.
19. E.C.D. and F.I.D. Relative Response Factors.

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TABLE I % Biodegradation after 52 and 100 days

Time	1242		1016	
	C_2	C_3	C_2	C_3
52 days+	88	76	96	91
100 days *	~95	~ 85	> 98	> 96

+ Analyses carried out using packed column method

* 100 day figures estimated by comparison of capillary column chromatograms for 52 and 100 days.

With added biphenyl

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TABLE II Half Lives of Components of Aroclor 1016 (days)

With added kinetics
Data from Fig III

Component	C_2	C_3
2,4' (From fig 4)		
* 2. 2'5' Tri-Di Chloro Biphenyl	26	~26
2. 2'4' " "	26	33
2 2'3' and/or 3 2'6' Di/tri " "	26	33
4, 2'6' " "	27	32
44' Dichloro "	25	27
2,2'4'6' and/or 25,2'6' Tetra Chloro Biphenyl	30	30
2 3'5' and/or 3,3'6' Trichloro Biphenyl	26	26
4 2'3' " "	26	26
* 3 2'4' " "	~26	~26
2 3'4' " "	26	26
2, 2'3'5' and/or 25 2'5' Tri/Tetra Chloro Biphenyl	26	26
2, 2'4'5' and/or 24,2'5' " " " "	26	26
3, 2'3'6' and/or 3, 2'4'6' " " " "	26	26
4,2'3'6' " " "	26	26
23, 2'4' " " "	28	24
2,2'3'4' and/or 23, 2'3' " " "	28	24
Unknown	26	24

* Peak off scale at attenuation used, half life estimated.

Designations correct with Fig VII MCS 1016

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Gas Chromatographic Analysis of Biodegraded Samples

Pentane extracts were obtained in a manner identical to that previously reported.

Method

A suitable aliquot (50 or 25 mls) of the pentane extract was evaporated to low volume (2 - 3 ml) in a Dreschel bottle on a water bath at 60°C using a nitrogen purge.

n Decane (0.1 ml) was placed into a tared specimen tube (13 mm x 45 mm) which was then reweighed. The concentrated pentane extract was pipetted into the decane followed by a small amount of pentane used to rinse the Dreschel bottle. The n pentane was then removed at room temperature again using nitrogen and the complete removal of pentane confirmed by reweighing the specimen tube.

Standards of Aroclor 1242 and 1016 were prepared as above by evaporation of 25 mls of a 1 ppm solution.

The rate of loss of any component was determined by measurement of the peak height of that component.

The concentration procedure was checked for reliability by running standards in duplicate - results were repeatable within ± 5%.

Gas Chromatographic Conditions

Column - 200' s/s capillary 30 thou bore (ex Phase sep.) coated with Apiezon L using the method of Malec E.J. ( ).

Temperature 210°C

Detector Ni^{63} (H&P) at 325°C

Carrier Argon/Methane 95/5 Input Pressure 5 p.s.i.

Internal Standard - None

Chromatogram Duration 90 minutes.

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TABLE 3. E.C.D. AND F.I.D. Relative Response Factors

Pk No	Cl No	E.C.D.	F.I.D.	E.C.D. / F.I.D.
5	2	0.02	0.01	2.0
6	2	0.06	0.09	0.7
7	3	-	-	--
8	2	0.38	0.83	0.5
9	3	0.61	1.08	0.56
10	3	0.37	0.47	0.79
11	3	0.40	0.47	0.85
12	3	0.42	0.25	1.68
13	2	0.03	0.21	0.14
14	4	0.05	0.05	1.0
15	3	0.18	0.17	1.1
16	3	0.19	0.14	1.35
17	3	1.0	1.0	1.0
18	3	2.10	1.71	1.23
20	3	0.54	0.40	1.35
21	4	0.23	0.29	0.79
22	4	0.31	0.32	1.0
24	4	0.35	0.27	1.30
25/26		-	-	-
27	4	0.13	0.07	1.9
28	4	0.29	0.20	1.45
29		0.25	0.12	2.08
30	3	-	-	-
31	3/4	0.16	0.32	0.50
0		-	-	-
32	4	0.08	-	-
35	4	0.35	0.32	1.10
36	4	0.17	0.14	1.21
37	4	0.08	0.08	1.00
38	4	0.02	0.03	0.66

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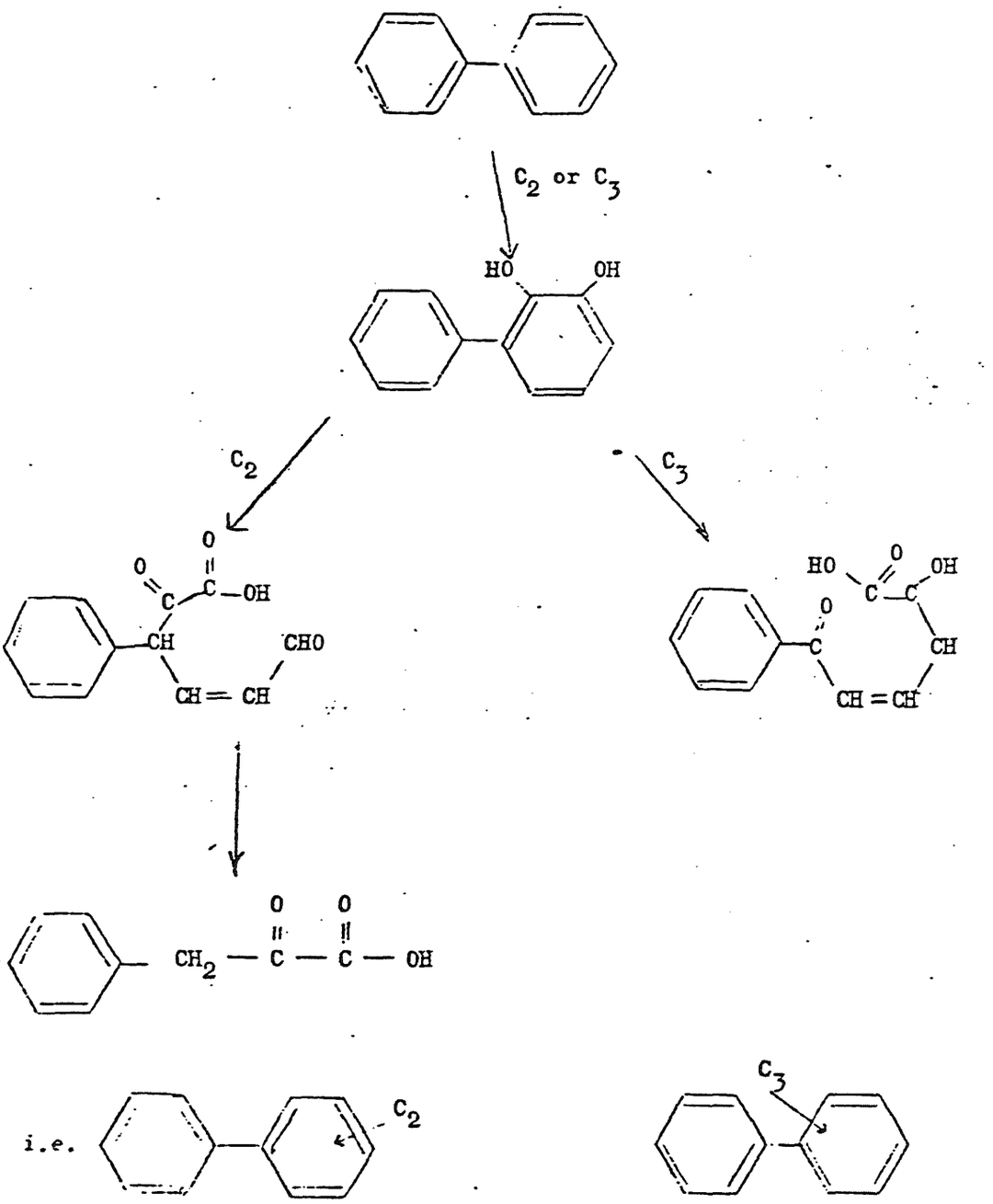
FIG. 9 Metabolic pathways for the biodegradation of biphenyl with C_2 and C_3



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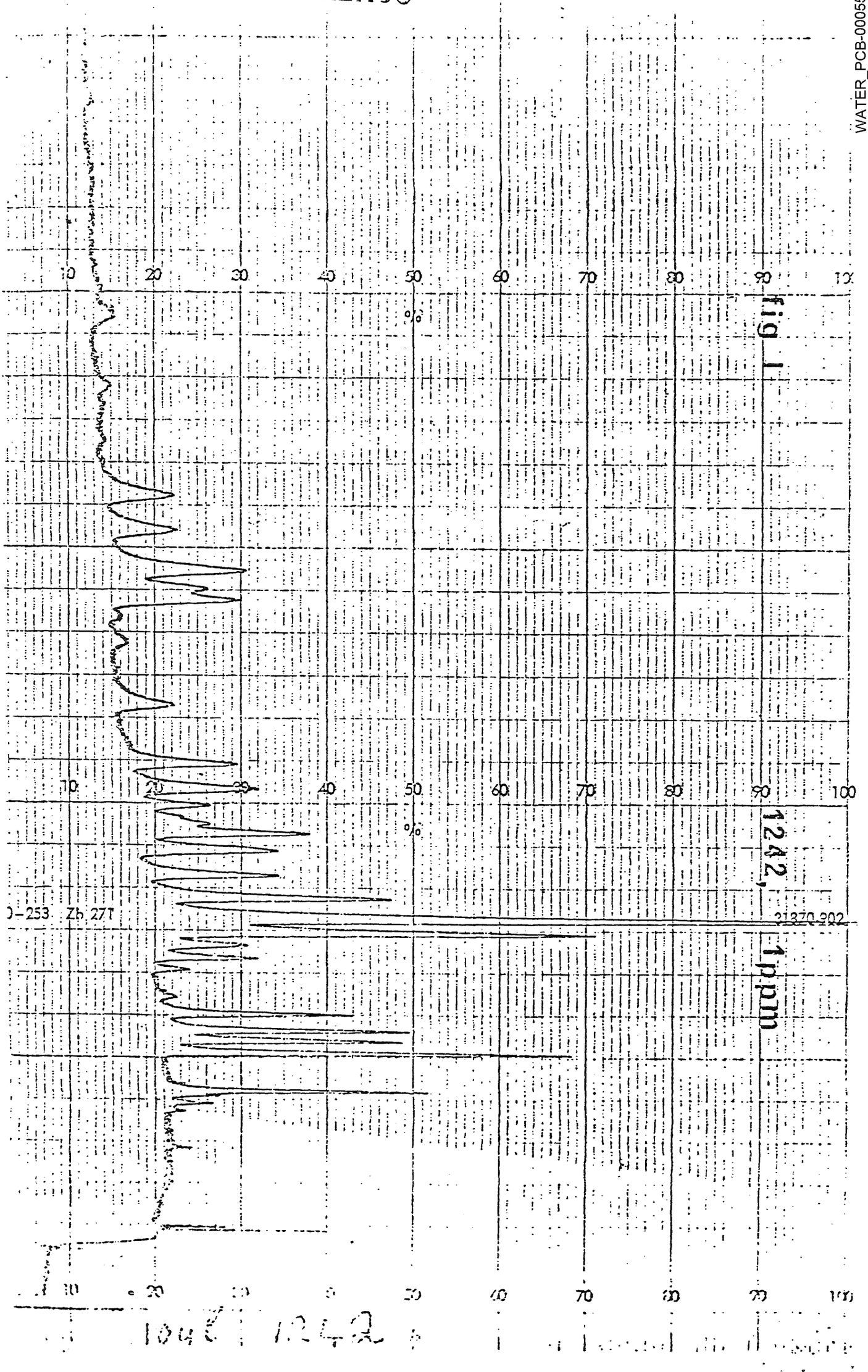

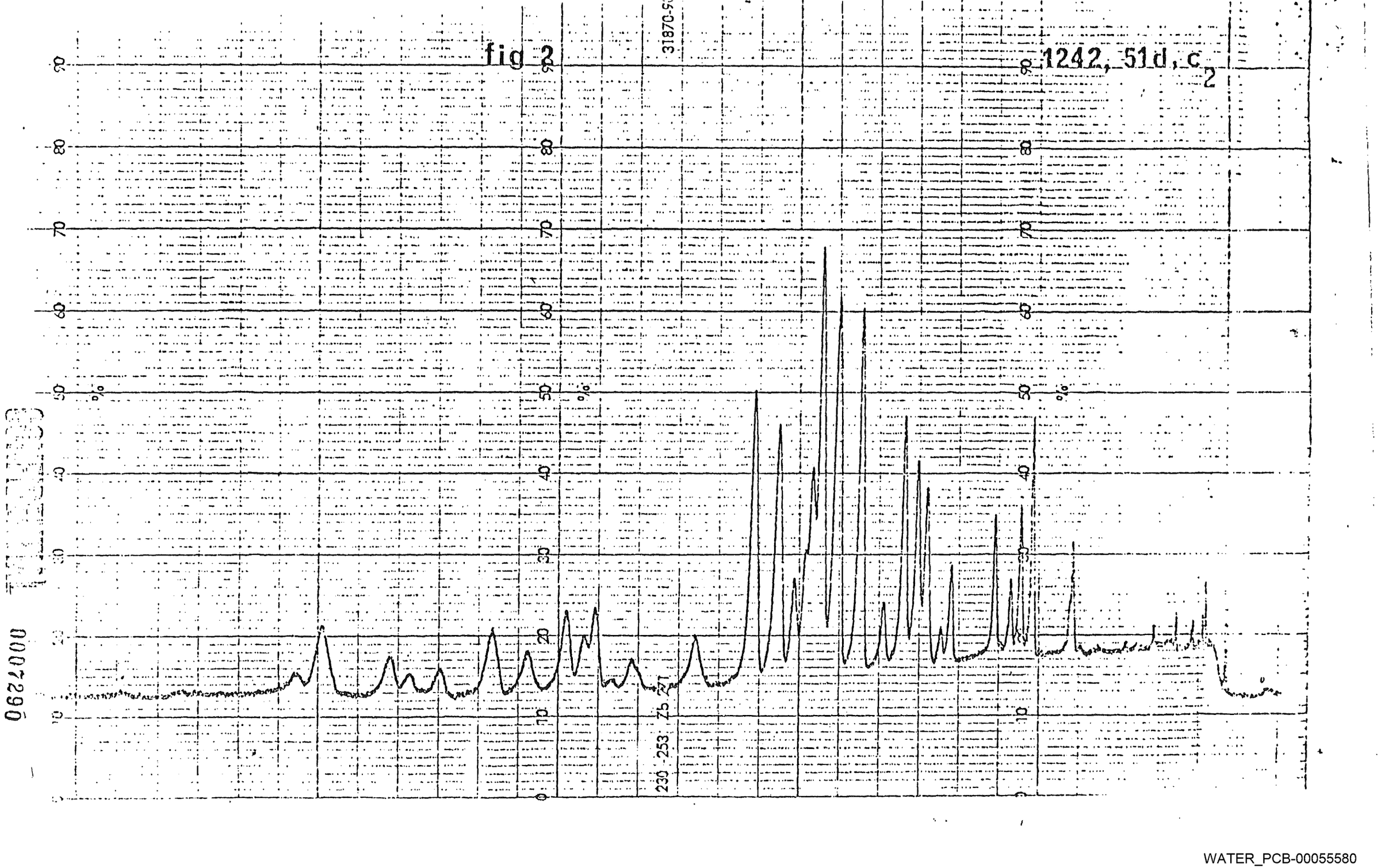

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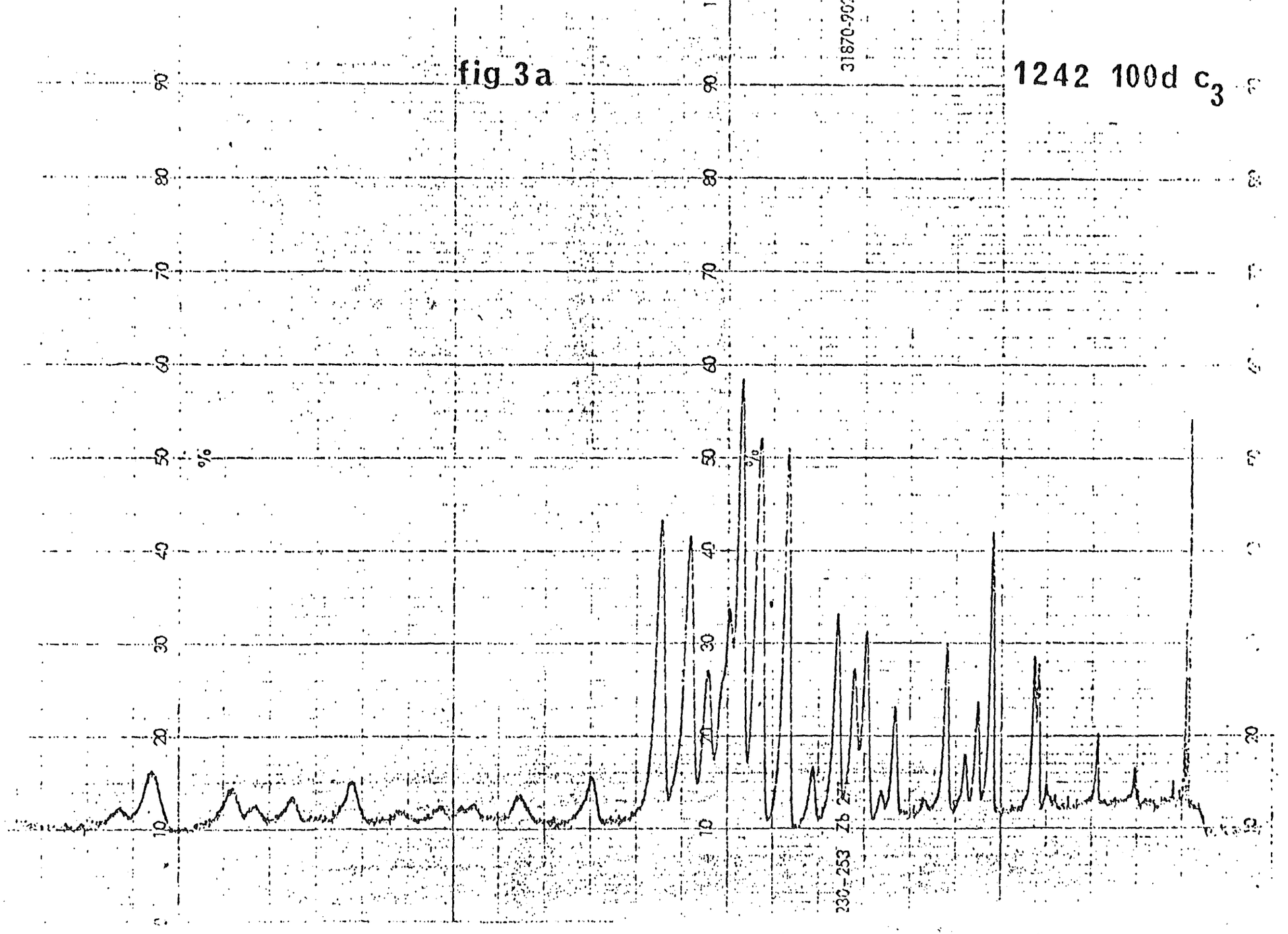
Fig 3 - 1242, 100d, C_2

WATER_PCB-00055582

fig 3a



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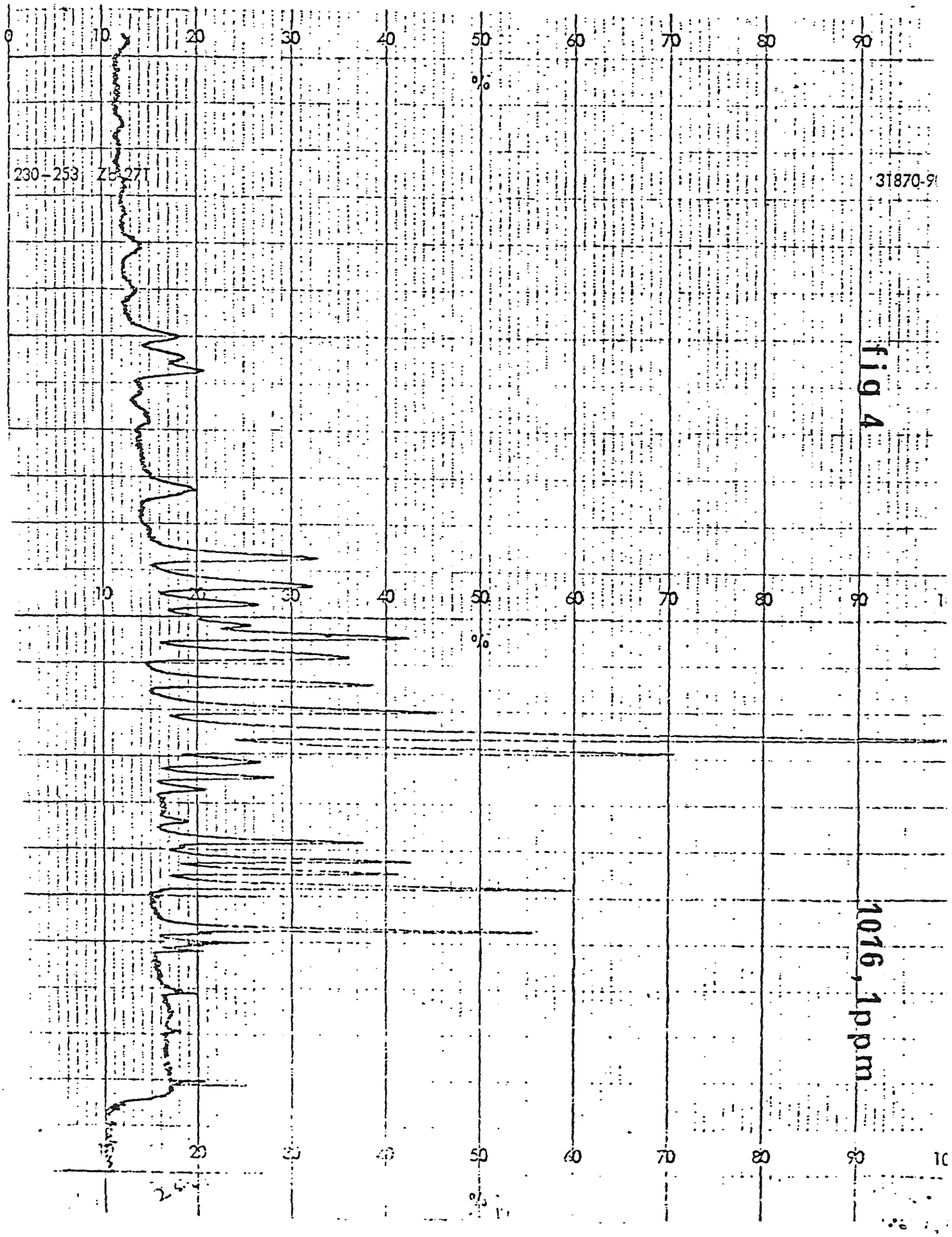

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WATER_PCB-00055585

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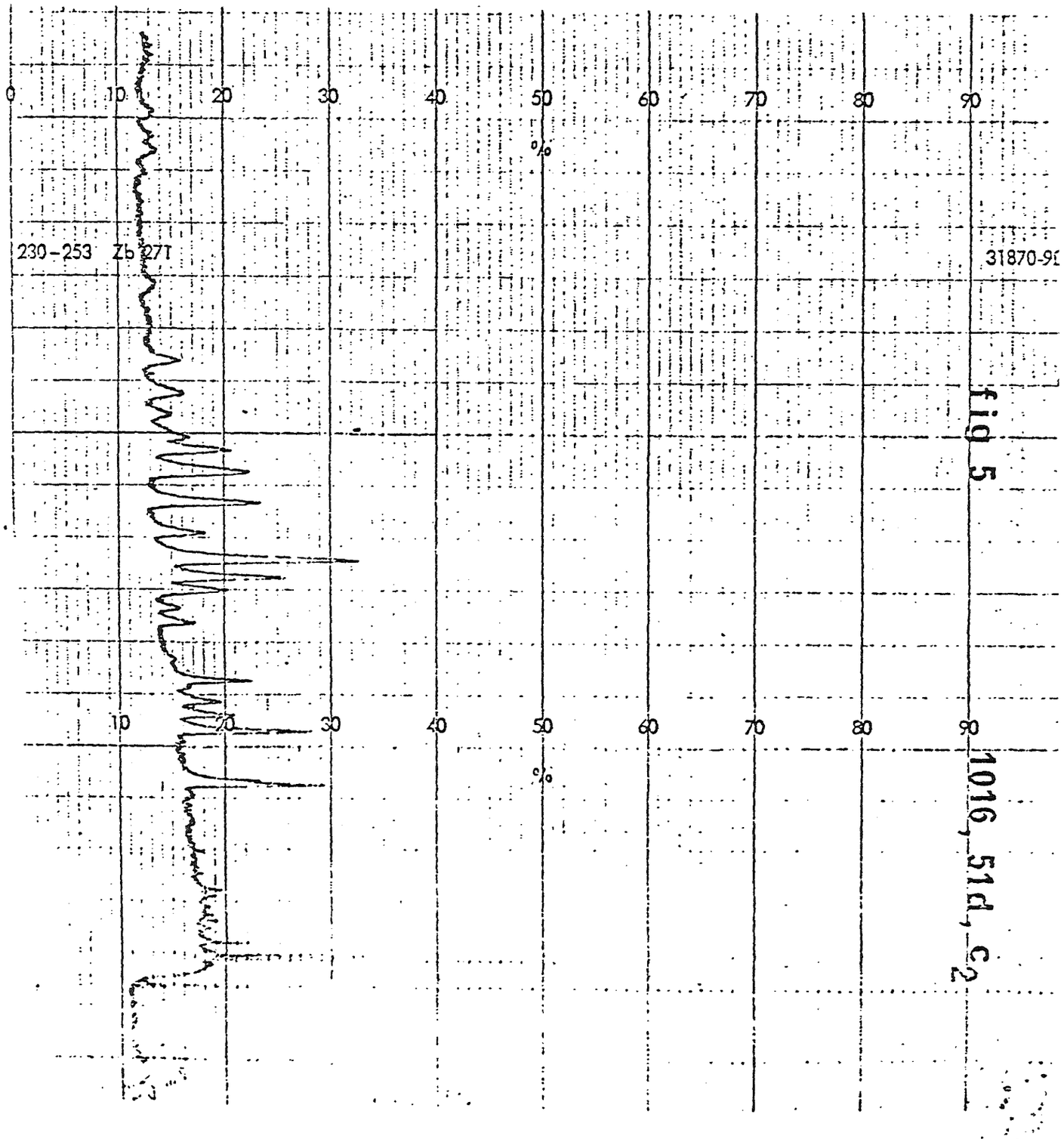

fig 5

1016, 51d, c_2

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Fig 6

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Fig 6a 1016, 100d, c_3

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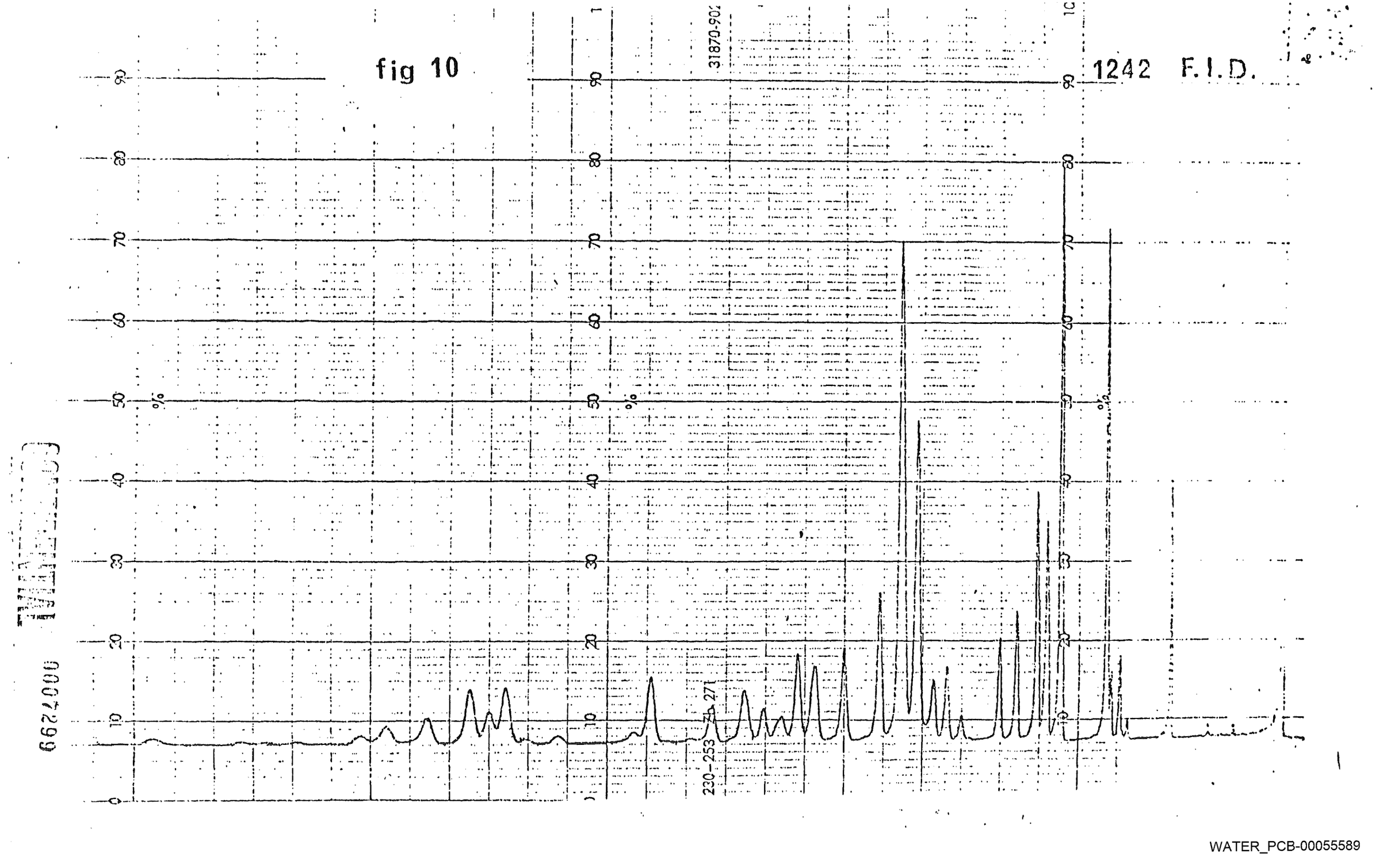
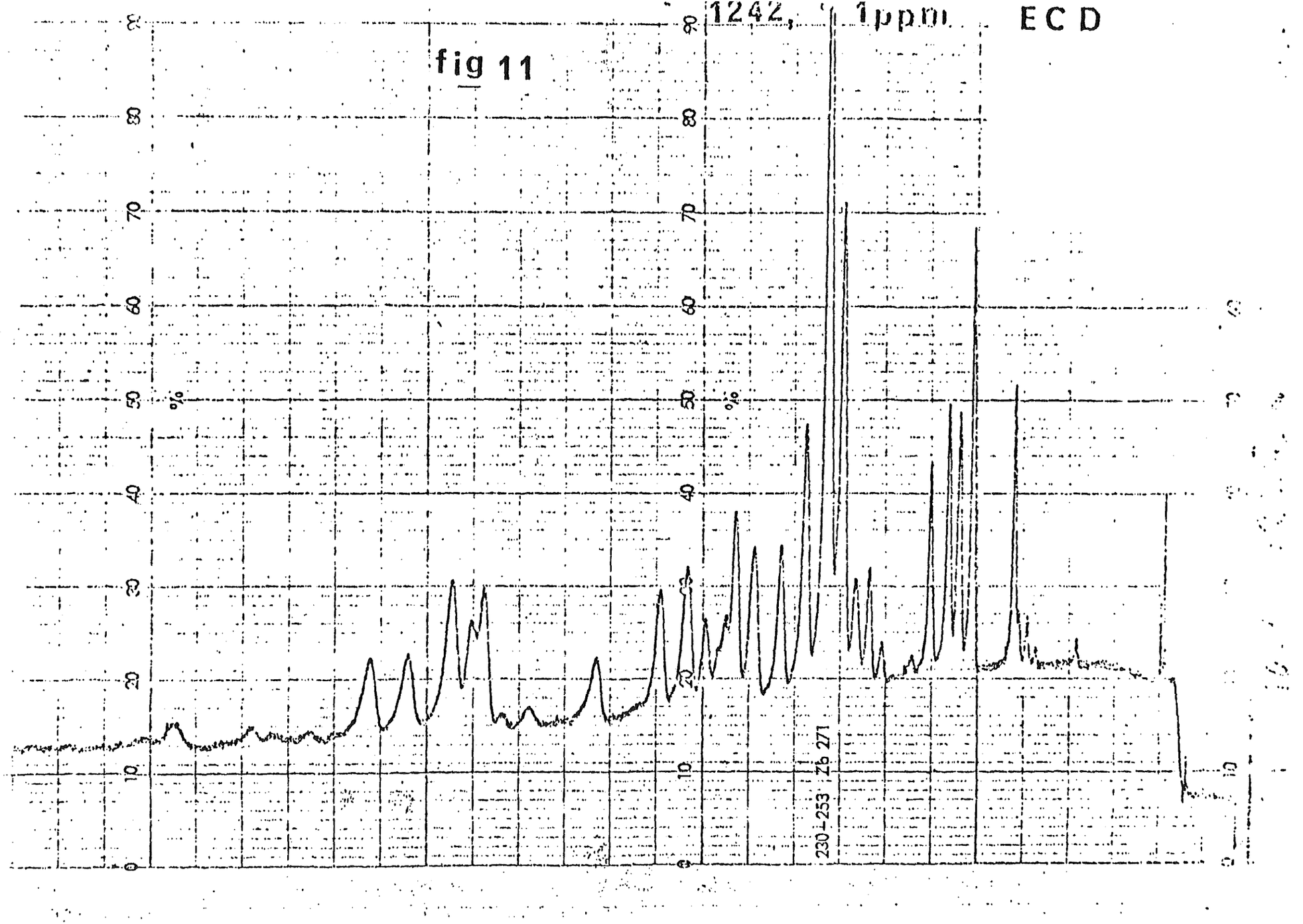
fig 10



WATER_PCB-00055589

fig 11



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With added ϕ_2

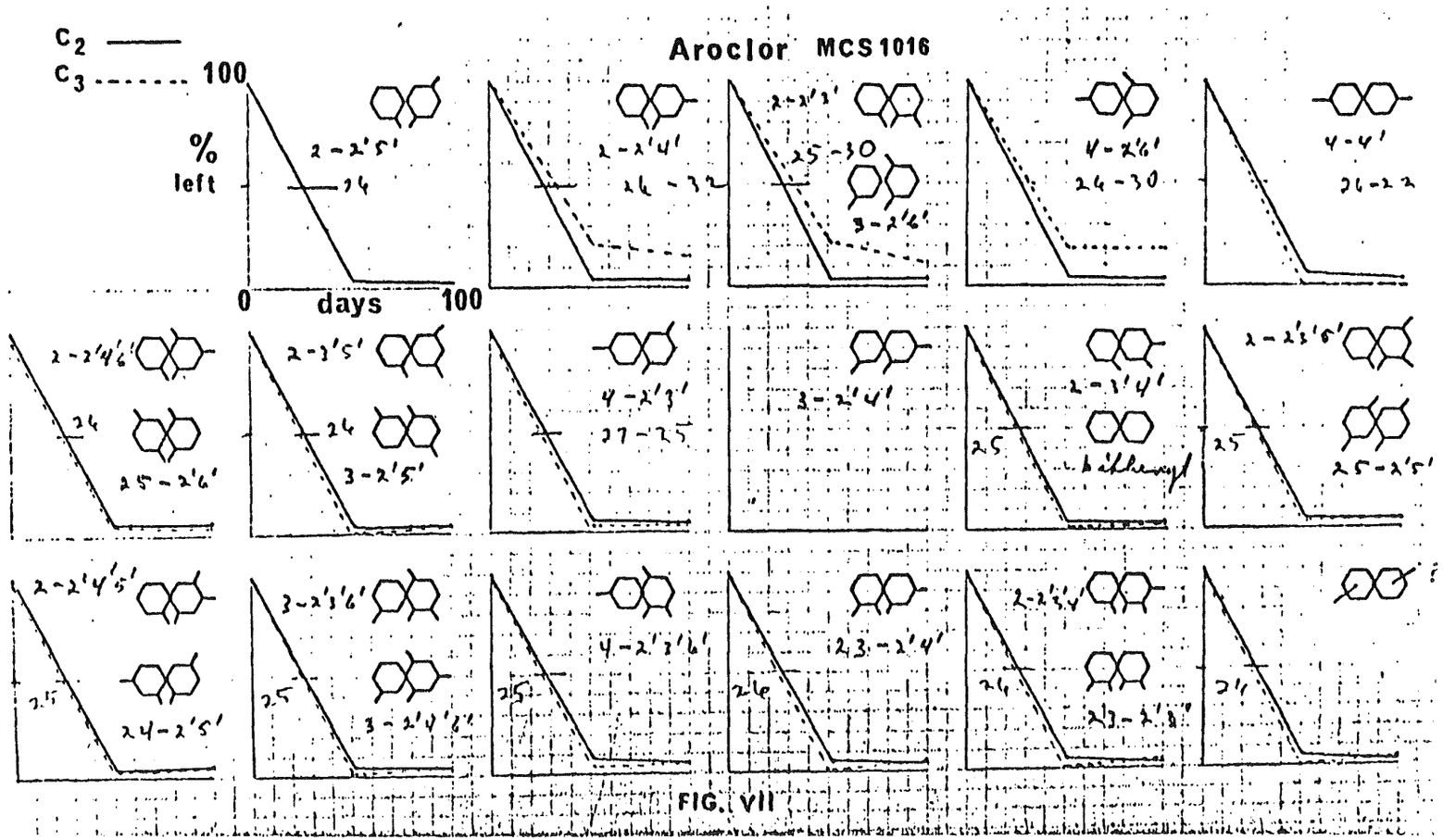

FIG. VII

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