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Isolation and cultivation of microbes with biodegradative potential

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1. Introduction

Microorganisms play a crucial role in the natural cycles of elements such as carbon, nitrogen, phosphorus and sulfur[54]. The carbon cycle, for example, refers to the largely photosynthetic fixation of CO_2, which is converted by e.g. plants, herbivores and carnivores into a multitude of complex molecules that are then degraded to CO_2 by fungi and bacteria, and this mineralization is termed biodegradation[54].

The development of enzymes for biodegradation is presumed to be by evolution during the 3×10^9 years that microorganisms have been in the biosphere[54], but this cannot be tested by examination of a fossil record (in contrast to e.g. the evolution of plant or vertebrate morphology) because of the paucity of the known record and the limited structure/function correlation in microorganisms. However, evolution of bacteria can be tested and demonstrated readily in the laboratory (due to their rapid rates of multiplication) and sets of extensive experiments have been done with biodegradative enzymes[13]. Long-term evolution in nature is seen as an on-going process which is presumed to occur not only vertically within a direct descendant pedigree but also horizontally through exchange of genetic information amongst otherwise independent vertical lines of development[4].

Man's purposeful application of biodegradation to clean up his wastes began in the middle of the last century[51] and was developed to a recognizable and functional sewage works early in this century[43]. Sewage works are usually capable of degrading a very wide range of naturally-occurring and synthetic (non-natural, alien or xenobiotic[35]) compounds[65]. But 'hard' detergents, which were not significantly degraded in sewage works and which then foamed in rivers and streams[11], raised public concern. Thus political pressure developed to eliminate these and other *recalcitrant* compounds, compounds that were not degraded in conventional sewage works.

Recalcitrance, however, is not a property like a molecular weight, which has a fixed numerical value; rather, it has become a label to indicate that a

compound is not degraded under particular conditions (see discussion in Slater and Somerville[59]). Recalcitrance is observed with natural products as well as xenobiotics[1], and the label can be readily misunderstood. Thus the highly toxic insecticide parathion (fig.) is normally short-lived in soil (about 30 days) but can be detected in some soils after 16 years[2]. 3,4,7,8-Tetrachlorodibenzodioxin (TCDD) on the other hand, has earned the label recalcitrant, because microbial metabolism is very slow (or nonexistant) and most claims for microbial degradation are attributable to artefacts[50]. Another case is that of melamine, which was found to be essentially non-biodegradable in 1937[55], then slowly degradable in 1964[32], but which is now known to be readily and completely biodegradable[15] and which has been patented as a nitrogen fertilizer for plants[3]. We presume that this apparent development of the ability to degrade melamine could represent evolution to utilize an industrial chemical, which is now used in large amounts[52].

The realization that at least some recalcitrant compounds are or become readily biodegradable has led to renewed interest in biodegradation as a means of removing problem (and presumably xenobiotic) pollutants. In this paper we shall describe the methodology and some successes, limitations and a failure to obtain organisms which satisfactorily degrade xenobiotic compounds.

2. *Aims, conditions and restraints*

The study of biodegradation covers a wide range of possible experimental approaches, reflecting the scope of activities of microorganisms in all the natural cycles of elements. We intend to discuss approaches to degrade single compounds which are presumably xenobiotic and are potential pollutants. It is essential to decide from the outset which approach or combination of approaches should be attempted. This means that the *aim* of the work must be clearly defined and the experiments *then* chosen to attain that objective.

The development of a project on biodegradation will be influenced by the funding authority, which may seek basic research or an industrial application where reaction rates and practicability are critical: the latter aspect is discussed by Leisinger[42]. In both cases, however, the primary question is 'Can we detect degradation of compound X by microorganisms?' And the answer should be 'Yes, $\geq 99\%$,' or 'No'.

The answer 'Yes' allows the study of:

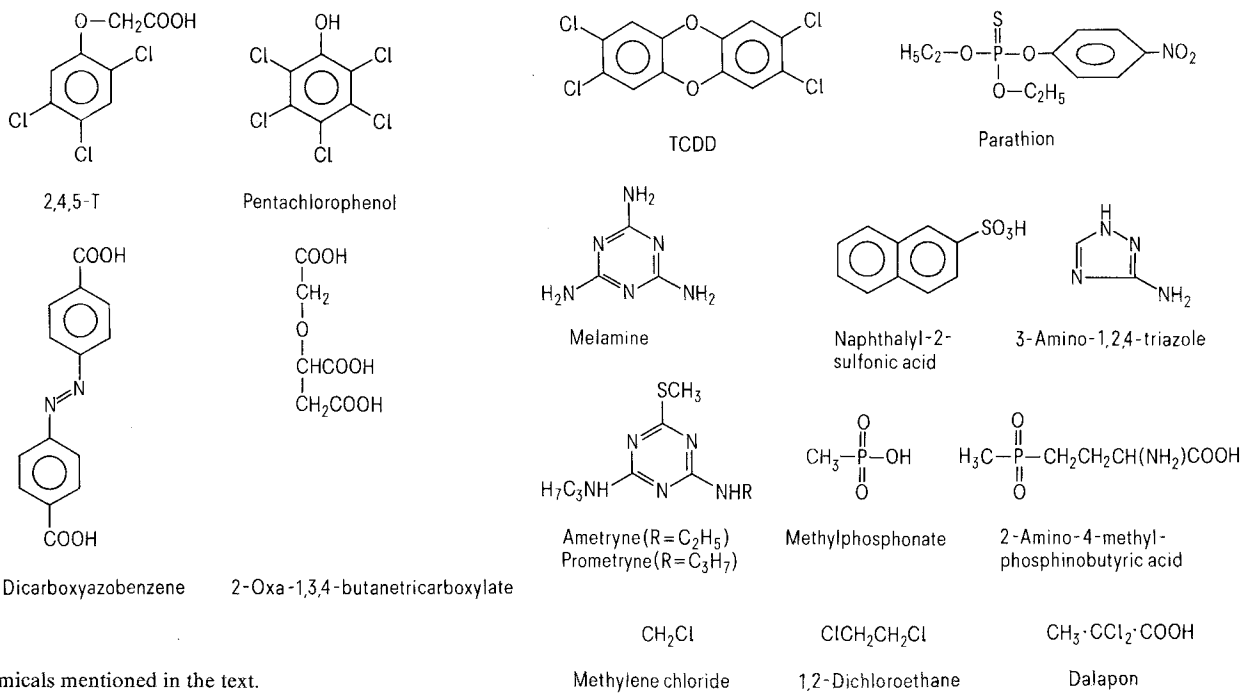
a) the microbiology of the process, be that at the level of the subtle interactions of mixed cultures[57], or of pure culture, or,

b) the chemistry and mass balance of the process, or,

c) the biochemistry of the biodegradative pathway, or,

d) the industrial application of the project.

The answer 'No' can have several meanings which are listed in the accompanying paper[42]. It is also important to use precise terminology: if 40% degradation occurs, does this mean that 100% of the substrate has been degraded and only 40% accounted for in the measured products, or is 60% of the substrate still present. This knowledge is of critical importance in deciding on the next experimental steps, if any, and demonstrates the need for good analytical chemistry from the start of the project.



Chemicals mentioned in the text.

A further important aspect of the aim is the concentration of the substrate to be studied. Should high concentrations (mM, representing waste water) or low concentrations (μM pollutant in soil water[68], nM hydrogen in soil water[14]) be used? A relevant answer to questions on utilization of the substrate will only be obtained if appropriate conditions are used[31]. For example, Conrad and Seiler[14] showed that the well-studied hydrogen bacteria (which grow with gas phases of about 80% H_2), did not in fact utilize hydrogen at the levels found in soil, in contrast to biological systems in soil which did.

We have previously discussed that cultures for industrial biodegradation must offer quantitative, fast and practical conversion of pollutant[16], and mentioned the advantages of pure cultures for studies showing complete conversion of the substance to defined, environmentally-safe products. The pure culture is more easily patented than an undefined mixture. The pure culture is also more easily and reproducibly grown in large scale than is a mixture[46]. These advantages of the pure culture need have little to do with the operation of a bioreactor used in practice to clean up waste. Bull[10] described how the herbicide dalapon is degraded in defined mixed culture with a higher specific activity than in pure culture. Furthermore, the disposal of multicomponent wastes will presumably require mixed cultures[31].

3. Enrichments

3.a. *Principle*

Microorganisms are ubiquitous[54], in that they are found in the atmosphere, natural waters and soil, even deep soil[69], as well as in sewage works and waste dumps. The range of activities of microorganisms is enormous[54]. And the problem is to obtain the organism(s) with the required degradative capacity – assuming such organisms exist. We believe the answer to be enrichment cultures, a very powerful technique dating from 1890–1900[64], but which seems to have been largely neglected for some years by those degrading wastes, perhaps because many textbooks illustrate its use for taxonomic purposes and because it was hoped to treat all chemicals in the conventional sewage works, the waste dump or the incinerator. Recent developments in the theory and practice of enrichments are to be found in Harder's excellent review[30] and Schlegel[53] and Veldkamp[66] have many useful suggestions. Manipulative techniques are described by Krieg[40].

The theory of the enrichment culture is startlingly simple. The compound to be degraded is supplied as the growth-limiting, and usually sole, source of an essential nutrient in a culture-medium. Only the organism(s) with the necessary degradative ability will grow significantly under these conditions and these organisms will outgrow the very large number of other organisms also added at the start of the experiment.

3.b. *Practice*

Before enrichment cultures are started, decisions should be made about four factors: growth medium, growth conditions, inoculum and analytical chemistry. Analytical chemistry warrants a separate section for discussion (see 3.c).

3.b.1. *Growth media and growth conditions*

Microorganisms consist of the elements C, O, N, H, P and S, varying in that order from near 50% to near 1% of dry weight, as well as ions (Mg^{++}, K^+) and trace elements[44], and these components at least must be supplied in the growth medium. A balanced growth medium for an aerobic heterotrophic bacterium is represented schematically in the table. A growth limitation, as required in the theory of the enrichment culture, is easily introduced by reducing one component relative to the others[16]. High purity of chemicals, especially of the presumed xenobiotic, is essential to prevent spurious enrichments[16].

Of the major constituents of the cell neither O nor H comes exclusively from a substrate (table), so neither is used as a limiting factor for growth when trying to degrade xenobiotics. It is usual to use xenobiotics as limiting carbon sources[59], which has the great advantage of removing the largest amounts of substrate per unit of growth (table). It is just as easy and may be advantageous to use the appropriate compounds as nitrogen, phosphorus or sulfur sources[15,17,20]. Campacci et al.[12] were successful in enriching for degradation of 3-amino-1,2,4-triazole only with the subtrate as a source of nitrogen, although carbon is theoretically available for growth. Cook and Hütter[15] made a similar observation with some s-triazines as substrates, though most of the latter substrates contain carbon only at the oxidation level of CO_2, which is not a growth substrate for heterotrophs. Cook et al.[20,21] similarly found that methylphosphonates are readily used as phosphorus sources but not as carbon sources: these experiments were done before the cleavage product was recognized as methane[28], which is lost from the aqueous phase, so a repeat in closed containers[62] is advisable. We have been unable to obtain organisms which utilize the herbicides ametryne and prometryne as carbon or nitrogen sources, but each compound is used readily as a sulfur source[17].

It has been normal practice, in enrichments to degrade pollutants, to use media, as seen in the table, designed to isolate aerobic organisms with simple nutritional requirements. Thus strains of *Pseudomonas*[7,15,17,20,21,38], *Alcaligenes*[49], *Klebsiella*[15,22] and coryneforms[12,60] are frequently isolated. These organisms represent only a tiny proportion of the known bacterial forms in Bergey's Manual[9], and if minor adaptations in the growth medium can make 'recalci-

trant' compounds biodegradable (see previous paragraph) we suspect that large reserves of degradative capacities are untapped because unsuitable growth media and conditions have been used (see table, right hand column, for suggestions). Veldkamp[66] gives valuable advice on the effects of conditions on enrichments. Fungi also offer a huge reservoir of poorly exploited degradative potential[25].

3.b.2. *The inoculum*

This is the source of the genes whose products will, one hopes, degrade the pollutant under study. The choice of inoculum is thus important.

Many successful enrichments are inoculated with material from a sewage works. Cook et al.[20,21] for example, isolated from sewage some bacteria degrading the so-called recalcitrant methylphosphonates[28]. But as there is a naturally-occurring methylphosphinate (2-amino-4-methylphosphinobutyrate[5]) we now suspect methylphosphonate to be a natural product (arising from the methylphosphinate) which has been tentatively identified in the Rhine[67], so it is not surprizing after all that methylphosphonate was degraded by so many isolates[21]. Kulla[41] found that an azo-compound (4,4′-dicarboxyazobenzene), also believed to be xenobiotic, was closely related to a natural product (4,4′-dicarboxyazoxybenzene), and Cain[11] describes a xenobiotic (2-oxa-1,3,4-butanetricarboxylate) that is a substrate for the citrate transport system and an ether lyase. Finally, Stanlake and Finn[60] obtained enrichments for the degradation of pentachlorophenol in all inocula tested.

Along with following the foregoing simple approach, one should also heed Schlegel's[54] advice and seek the organisms where they are most likely to occur. In the case of the herbicides ametryne and prometryne, enrichments were only successful from soils with very long exposure to s-triazine herbicides; short or zero exposure yielded no enrichments[17]. In a similar manner, Stucki[61] obtained organisms able to degrade methylene chloride and 1,2-dichloroethane only from old dumps of chemicals. We presume this phenomenon to represent an evolution of the bacterial population to utilize these chemicals. Other sources of inocula, which may have evolved degradative enzymes, are drains from the production line and the appropriate industrial sewage works. In an attempt to improve the evolutionary development in their enrichments for the degradation of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), Kellogg et al.[37] chose a complex inoculum in which not only material from waste dumps but also defined strains harboring plasmids were used. There was apparently no comparable control without added plasmid-carrying strains or a test of homology between the plasmids in the degradative culture and the initially added plasmids, but the experiment was successful in yielding a culture utilizing 2,4,5-T as a carbon source for growth. Veldkamp[66] points out the value for enrichments of the enormous variability in soil, thus the larger the number of sensible inocula available, the more likely is the chance for a successful enrichment.

A theoretical and schematic balanced growth medium[a] for a mesophilic, aerobic, heterotrophic[b] bacterium

Component or condition[c]	moles ±30% per unit of growth[d]	Comment	Alternative conditions
C	700	Value varies widely with redox value of substrate	More carbon is necessary under anaerobic conditions because an organic product is expected
O		In part from water, O_2 and perhaps substrate	Variation in $[O_2]$ will lead to enrichment of different organisms
N	80	If enriching with a nitrogen limitation, eliminate N_2	
H		Derived in part from H_2O and in part from substrate	H_2 can be used as an energy source
P	3	It is difficult to make solid media free of contaminant P	
S	1		Many autotrophic and anaerobic reactions known
Mg, K, traces			
Buffer		The concentration must suffice to neutralize metabolic products	
pH		May be controlled by pH-stat	Variation of set pH will lead to different enrichments
Gases		In part O_2	Variation of the gas phase will also alter enrichment
Temperature		20–42 °C	This parameter may also be varied

[a] The data shown represent a balanced medium. To obtain a nutrient limitation as required in the theory of enrichments, the amount of the one component which is supplied in the compound to be degraded is lowered relative to the others. [b] Mesophiles grow between 20–42 °C; aerobes utilize O_2 as terminal electron acceptor; heterotrophes obtain cellular carbon from organic compounds[54]. [c] Each nutrient is represented as an element, which is of course present as an organic or inorganic compound (except e.g. O_2) in a real growth medium. [d] The components would have to be differently balanced for e.g. anaerobic or autotrophic growth medium[40]. Marine organisms and thermophiles would require other conditions as would fungi.

3.b.3. *Batch and continuous culture*

Having arranged the initial factors, the decision must be made whether to use batch culture or continuous culture, each of which has advantages and disadvantages.

Batch culture is done simply in test tubes[15,17,20,21] or shake cultures[62], which allow many individual experiments to be set up with little effort or expense. This is the obvious starting point in enrichments and it can be the last stage in many enrichments from continuous culture[29]. The batch culture has the enormous advantage of requiring very little of the com-

pound under study, as the pure compound is often available in only low amounts. The disadvantages appear if the compounds are poorly soluble or highly toxic to microorganisms. A further drawback is the lack of strong selective pressure on newly-arising mutant strains to outgrow the parents, because few organisms are present relative to the mutation rate (10^{-8} per base pair and generation[54]) and growth is limited.

The continuous culture complements the batch culture. It enables the experimenter to exert great selective pressure to obtain utilization of the compound under study[58]. In the advanced technique of Knackmuss' group[7,29] not one but two carbon sources are supplied, the first is a growth substrate (e.g. naphthalene) that is presumably metabolically related to the pollutant and is present at low concentration to maintain a steady growing population over a prolonged period (thus maintaining a steady supply of mutants), and the second carbon source is the pollutant (e.g. naphthalyl-2-sulphonic acid) which is supplied in increasing amounts. An increase in cell density implies utilization of the pollutant and the first substrate is removed. Continuous culture further allows the use of low concentrations when the chemical is sparingly soluble[26,41] or toxic. The disadvantages lie in the amounts of substrate consumed (assuming this is a problem), the large amounts of apparatus required per culture and the correspondingly small number of experiments that can be started, and a tendancy to produce organisms which grow very slowly (see 3.b.5.).

3.b.4. *Procedure and problems with batch enrichments*

If the series of decisions following the aim of the project has led to a batch enrichment approach, the inoculum (better, inocula) is washed to reduce contaminant nutrients and the experiments (including negative and positive controls) are started under non-sterile conditions. The first culture seldom gives unambiguous data, despite the washed inoculum, and there is often little difference in turbidity between positive and negative controls due in part to the presence of clay minerals. The enrichments are subcultured into homologous medium. In this subculture it is usually possible to distinguish which cultures are growing well and which are never better than the negative control and may be discarded. The positive enrichments are subcultured about twice more to confirm that the activity can be maintained and that it is not an artifact due to catalysis on clay surfaces. Parallel to the enrichments, controls for substrate stability are examined and substrate disappearance in all cultures is measured. We have observed unexplained growth without substrate utilization, which stresses the importance of analytical chemistry to corroborate the deduction of degradation made by observing growth in an enrichment.

An enrichment culture which quantitatively degrades the substrate is the starting point for work with mixed cultures or for an attempt to isolate pure cultures. If conversion of the substrate is incomplete or too slow, it is important to check back with the set aims of the experiment and decide whether the experiments should be continued or another enrichment attempted.

The isolation of a pure culture from an enrichment, be it from a batch or from a continuous culture, typically starts by streaking the culture on a solidified non-selective medium and transferring a specimen of each colony type to sterile selective liquid medium. Experimentally, the simplest result is that growth is observed in a few of the liquid cultures and is accompanied by substrate disappearance. The liquid culture is again streaked on plates of non-selective medium and a colony is transferred to sterile, selective liquid medium. When three successive plates yield colonies of identical, homogenous morphology, the culture is considered pure and can be stored safely (see 3.b.6). The use of non-selective plates is to prevent the carry-over of persistent contaminants often found if selective plates are used[24] whereas the alternate use of selective and non-selective media is to reduce the chance of losing the degradative capacity on successive non-selective plates[20].

It is not uncommon that, after plating and picking colonies, no growth occurs in selective media. There are several possible explanations for this phenomenon.

a) *The isolated organism never contained the degradative ability.*

b) *The degradative organism grows much more slowly on plates than do the contaminants,* a problem that is easily solved by a longer incubation.

c) *The isolated organism has lost the degradative ability during development on the plate.* It is then possible to try repeatedly to pick from fresh non-selective plates, or to use selective plates or to isolate by terminal dilution. The experience of Kilbane et al.[38] with the degradation of 2,4,5-T suggests that, in early experiments, the activity was very easily lost and that a more stable mutant strain was finally isolated.

d) *The degradative organism does not grow on the plates used.* There are some habitats from which very few organisms in the population can be plated[69]. It is possible to test plates of different composition, in case a component(s) is toxic[66], but here too, the solution seems to lie in terminal dilution, as done[61] for the organism degrading 1,2-dichloroethane[62]. The bacterium is presumably pure when there is no growth on nutrient agar and the culture is microscopically homogeneous.

e) *The degradative organism grows only in mixed culture.* In this case it may be possible to supply externally the growth requirement otherwise met by

another organism in the mixture. Harder[30] and Slater[57] give specific examples of this phenomenon. Cook and Hütter[17] observed that addition of vitamins speeded growth of the mixed culture, and by adding vitamins to selective and non-selective media they were able to obtain pure cultures of bacteria utilizing ametryne as a sulfur source.

f) *Degradation occurs only in mixed culture.* Some examples of this problem are given by Harder[30]. In the case of parathion, growth with parathion as sole carbon and energy source was only obtained in mixed culture[48]: Daughton and Hsieh[26] obtained growth in a defined two-component system and the first metabolic reaction, hydrolytic cleavage releasing p-nitrophenol, was found to be in one organism and the degradation of p-nitrophenol in the other. Munnecke and Fischer[47] found the expression of parathion hydrolase to be constitutive and they could grow the pure culture on a large scale and obtain the degradative enzyme in large amounts for practical use[46].

Although with hindsight explanations can be found for the phenomenon of failure to pick successfully from plates, the experimenter does not know which, if any, applies to his case. We suggest firstly a longer incubation of the plates, then the addition of vitamins or amino acids, then different solidifying agents, or isolation on selective plates, terminal dilution and repeated platings over weeks.

The phenomena of cometabolism are not discussed here because the term has too many meanings[34] and tends to elude rather than elucidate a problem, because an author usually does not have to define which, if any, of these meanings he has in mind.

3.b.5. *Procedure and problems with continuous culture enrichments*

Apart from the purely physical advantage of using continuous cultures with substrates of low aqueous solubility[26,41], the ability to obtain organisms capable of growing at low substrate concentration[30] and the ability to maintain defined conditions (as against the constantly changing conditions in batch culture[66]), the enormous advantage of the continuous culture is the opportunity to exert great pressure to select organisms that have mutated to degrade novel compounds[7,29,56] as described in section 3.b.3. In the case of the successful enrichment for the utilization of naphthalyl-2-sulphonic acid as sole carbon and energy source, an organism, *Pseudomonas* sp. strain A3, was successfully isolated[7]. This strain has a relatively high specific growth rate ($\mu = 0.17\ h^{-1}$) compared with other organisms derived from continuous cultures[38,41] (e.g. $0.07\ h^{-1}$, $0.09\ h^{-1}$) but this is still slow compared with some older laboratory strains like *Escherichia coli* growing with glucose[33] ($1\ h^{-1}$) or *Acinetobacter calcoaceticus* utilizing benzoate[18] ($0.9\ h^{-1}$). But whatever criticisms are raised, the continuous culture as used by Brilon et al.[7] has yielded organisms able to degrade and utilize a variety of pollutants as carbon and energy sources[38,41].

3.b.6. *Storage of biodegradative strains*

It can be very time-consuming to obtain a degradative organism, so the organism must be protected against loss by safe storage. Storage on selective medium is almost mandatory, considering the ease with which activities can be lost under non-selective conditions[7,38]. Slants protected from water loss can be stable for months, but this must be tested. Freeze-dried cultures are usually stable for many years, but this too must be tested, not only as a test of viability and purity but also to ensure that the degradative ability has been successfully retained. If slants are impossible, either because the organism does not grow or because selectivity is not possible due to contaminants, liquid cultures (usually at 4 °C) may display remarkable stability, but then a permanent reserve of organisms is an essential.

3.c. *Analytical chemistry*

Microbiology is in part intuitive, especially in the isolation of unknown organisms from enrichment cultures. Intuition, however, is not proof of biodegradation; there is no substitute for direct analyses and we have repeatedly had to report that claims of biodegradation for many compounds are unsupported by experimental data[15,17,20,23].

The first quantity to be measured is growth. We use protein as an assay for growth because it eliminates trivial interferences in assays of turbidity or dry weight due to storage polymers, and the assay then allows all data to be on a protein base, as is any subsequent enzyme research[36]. If carbon-limited cultures are being studied, turbidity can be a good method[39], but it must be calibrated to dry weight or protein if it is to be of value because turbidity (unlike absorbance) varies enormously with instrument and cell type. Stanlake and Finn[60] use turbidity as an assay of dry weight and give the growth yield with pentachlorophenol as sole carbon and energy source in units of dry weight. This we calculate to be about 4 g of protein/ mol of C (using standard conversion factors[45]), a normal value[16]. Kilbane et al.[38] use turbidity without calibration but they do give viable counts which we calculate to represent about 2% of the added carbon (using standard conversion factors[45]): however, the authors describe release of 100% of the organochlorine as chloride, which was tentatively identified. We have found protein growth yields an excellent method of evaluating substrate utilization, especially with a control experiment done in parallel[15,17,20,23].

An essential complement to the growth yield is an adequate and preferably sensitive, specific determin-

tion of the substrate: if necessary, an assay must be developed[6,8,19,27]. The proof of substrate disappearance, with relevant controls, and coupled to quantitation of growth is strong evidence of biodegradation[15,17,20,23,60]. These data allow mass balances to be drawn, which immediately reveal any major discrepancy. Thus Philippi et al.[50] followed up older arguments, that TCDD was degraded in soil, and discovered that the disappearance of TCDD was not due to metabolism but largely to binding to soil; the 'missing' TCDD was released by exhaustive extraction techniques. Neither an assay of growth alone nor substrate disappearance alone is proof of biodegradation.

The burden of proof that a reaction occurs lies with the experimenter. The reaction product may be logical (i.e. an attractive hypothesis), but only adequate chemical identification will give substance to hypotheses. Brilon et al.[8] give a good example of thorough identification of metabolites which gave substance to arguments on reaction mechanisms. And chloride ion may be the logical product from an organochlorine compound, but Stucki et al.[63] confirmed the tentative identification of chloride (with an ion-specific electrode) by using two other independent methods; the organic product was also conclusively identified.

4. Conclusions

We can review major successes like the degradation of 2,4,5-T[38] or pentachlorophenol[60] or of compounds which represent 7–15% of the pollution in the Rhine[7,8] but there are problems for which biodegradation is inapplicable and others that are unsolved. Inorganics contribute about $\frac{1}{3}$ of chemical production[35], so there are presumable wastes from $\frac{1}{3}$ of chemical production for which biodegradation is irrelevant. And TCDD is currently degraded at a negligible rate by bacteria, in part because it is so insoluble in water, in part because of its ready binding to surfaces.

The foregoing discussions have shown that bacteria can be obtained to degrade a wide range of potential or actual pollutants. We suspect that a much wider range of organisms has not yet been exploited: adaptable methods exist to obtain new organisms and we have tried to indicate how to use these methods. The cultivation of these degradative organisms is essentially identical to that in classical microbiology, but, given the sublte nature of the problems of contemporary pollution, the powerful contemporary methods of analysis are necessary to test the mass balance during disappearance of pollutant.

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