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BACTERIAL DEGRADATION OF ALDER (*ALNUS GLUTINOSA*) LEAVES IN A FRESHWATER STREAM

A Thesis submitted for the Degree of Doctor of Philosophy in the University of London.

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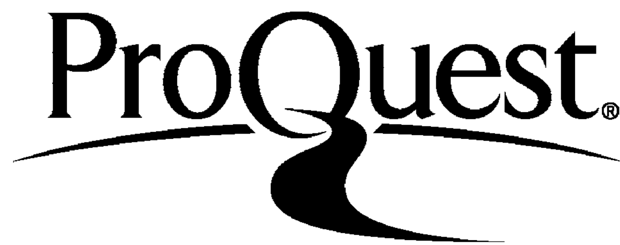

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ABBREVIATIONS

Abbreviation		Meaning
cm	-	centimetre/s
CA	-	chitin agar
cmc	-	carboxymethylcellulose
fig/s	-	figure/s
G+	-	Gram positive
G-	-	Gram negative
hr	-	hour/s
HSD	-	Honest Significant Difference
l	-	litre/s
M	-	molar or molarity
ml	-	millilitre/s
mm	-	millimetre/s
N	-	normal
NB	-	nutrient broth
NG	-	no growth
nm	-	nanometres
no.	-	number/s
OA	-	oatmeal agar
PA	-	pectin agar
PAA	-	Pseudomonas Aeromonas Agar
Ps.	-	Pseudomonas
PSS	-	peptone succinic acid agar
RBB	-	remazol brilliant blue
ref/s	-	reference/s
SEM	-	scanning electron microscope/ microscopy
sp	-	species (singular)
spp	-	species (plural)
TEM	-	transmission electron microscope/microscopy

TSBA	-	trypticase soy broth agar
µg	-	microgram/s
µm	-	micrometres
UV	-	ultra violet
VDD	-	vapour diffusion dehydration
v/v	-	volume for volume
w/v	-	weight for volume
YEME	-	yeast extract malt extract agar

ABSTRACT

A sequential colonisation by bacteria of decaying alder leaves in the River Bourne and of sterile alder leaf discs *in vitro* was demonstrated by SEM. Enumeration of colonising organisms by fluorescence microscopy confirmed SEM observations that colonisation commenced within 24h and that numbers of bacteria then increased slowly over the first week of incubation, then declined and finally stabilised for the remainder of the decay period. Colonisation was divided into 4 phases: initial colonisation, establishment, instability, stabilisation.

Organisms colonising alder leaves were isolated by direct plating and enrichment culture and characterised by standard morphological and biochemical testing, API 20NE strips where appropriate, and fatty acid profiling. Thirteen different genera were isolated and these were *Xanthomonas*, *Pseudomonas*, *Aeromonas*, *Alcaligenes*, *Erwinia*, *Bacillus*, *Rhodococcus*, *Escherichia*, *Cytophaga*, *Proteus*, *Janthinobacterium*, *Streptomyces* and *Klebsiella*. Morphologies observed under SEM of pure cultures of these genera on sterile alder leaf discs were similar to those observed *in vivo* and a known mixture of bacteria colonised sterile alder leaf discs *in vitro* in a pattern similar to that observed *in vivo*.

A survey of production of pectinases, cellulases and xylanases by representatives of each genus showed that

representatives of all class of enzymes were produced although not all isolates were shown to produce the appropriate enzymes. Bacterial activity in pure cultures and in mixed culture with the aquatic Hyphomycete *Tricladium splendens*, however, showed that with the exception of *Aeromonas* significant reductions in dry weight of alder leaves were affected by all isolates. The largest weight losses were caused by Streptomyces sp , the mixture of bacteria and *T. splendens* and *Cytophaga* all of which possess cellulolytic enzymes. This suggests tht cellulolytic activity accounted for the greatest reduction in dry weight.

TABLE OF CONTENTS

	Page
Abbreviations	1
Abstract	3
Table of Contents	5
List of figures	10
List of tables	14
Introduction	19
Materials and Methods	49
1. The site	49
2. Leaves and leaf packs	49
3. Leaf pack construction	49
4. Collection and processing of leaf packs	50
5. Media, stains and reagents	50
i Peptone succinic acid medium	51
ii Oatmeal agar	51
iii TA medium	51
iv Chitin agar	52
v Pectin agar	52
vi Leaf homogenate agar	53
vii Paste medium	53
viii Yeast-extract-malt-extract agar	54

ix Tap water agar 54

x Enrichment medium 54

xi Tryptone soy broth agar 55

xii Dye's medium C 55

xiii Tryptone broth 56

xiv Simmon's citrate agar 56

xv Starch agar 56

xvi Gelatin medium 57

xvii Tryptone yeast extract broth 57

xviii Trypticase soy broth agar 57

xix Yeast extract agar 58

xx Hugh and Liefson's medium 58

xxi McBeth's cellulose ammonium sulphate 58

xxii Gram's stain 58

xxiii Cell wall fatty acid extraction reagents 59

xxiv Xylanase assay medium 59

xxv Sodium polypectate gel 60

xxvi Pectate lyase induction medium 60

xxvii Pectin methylesterase induction medium 61

xxiii Cellulase induction medium 62

6. Sampling from packs 62

a) Culture 62

i Direct plating 62

ii Enrichment techniques 63

iii Direct isolation by plating 65

iv Isolation of specific genera 65

7. Direct viewing of colonised organisms 67

i Scanning electron microscopy 67

ii Fluorescence microscopy 71

iii Direct enumeration by fluorescence microscopy of river water organisms colonising sterile alder leaf discs *in vitro* 71

8. Identification of Isolates 73

a) General testing 73

i Colony pigmentation 73

ii Cell morphology and arrangement and motility 73

iii Gram stain 74

iv Carbohydrate fermentations 74

v Anaerobic growth 74

vi Catalase production 75

vii Oxidase production 75

viii Indole production 75

ix Citrate utilization 75

x Starch hydrolysis 75

xi Gelatin liquefaction 76

xii Oxidative-fermenative metabolism 76

xiii Flagellar number and insertion 76

xiv Digestion of filter paper strips 77

b) Testing of Putative *Xanthomonas* spp 77

i Spreading growth pattern 77

ii Carbohydrate fermentation 77

iii Bacteriolytic activity 77

iv Polymyxin B sensitivity testing 78

c) Cell wall fatty acid extraction and profiling 78

d) APl 2ONE Bacterial Identification strips 80

9. Succession of bacteria on decaying alder leaves - *in vitro* study 81

10. Leaf Polymer content 87

11. Substrate utilization 90

a) Enzyme induction 91

i Pectate lyase 91

ii Pectin methylesterase 92

iii Cellulase 92

b) Assay Methods 93

i Pectate lyase 93

ii Pectin methylesterase 94

iii Cellulase 94

c) Dry weight reduction 95

Results 97

1. SEM study of succession of morphotypes on decaying alder leaves in freshwater. 97

2. Enumeration of bacteria colonising decaying alder leaves. 118

3. Characterisation 125

4. *In vitro* colonisation of alder leaves by a known mixture of bacteria. 213

5. Microscopy - general observations 223

6. Enzyme survey 223

- i Pectinases 235
- ii Xylanases 244
- iii Cellulases 245

7. Loss in dry weight of alder leaves as a result of microbial activity. 247

Discussion 252

Conclusions and future work 284

Acknowledgements 285

References 287

Appendix 309

LIST OF FIGURES

Fig. 1 Construction of cellophane wick apparatus for selective isolation of microorganisms.

Fig. 2 Construction of apparatus for vapour diffusion dehydration of samples for SEM.

Fig. 3 Construction of a sidearm with vent in a fermenter to drain excess water build-up in the event of air-locking.

Fig. 4 Typical growth obtained on PAA from spread plate inoculation with a defined mixed culture.

Fig. 5 Typical growth obtained on CA from spread plate inoculation with a defined mixed culture.

Fig. 6 Typical growth obtained on NA from spread plate inoculation with a defined mixed culture.

Fig. 7 Typical growth obtained on MacConkey agar from spread plate inoculation with a defined mixed culture.

Fig. 8 Typical growth obtained on YEME from spread plate inoculation with a defined mixed culture.

Fig. 9 Typical yeast cells observed in vivo on dried alder leaves.

Fig. 10 Typical cocco-bacilli observed in vivo on decaying alder leaves.

Fig. 11 Typical intermediate length bacilli observed in vivo on decaying alder leaves.

Fig. 12 Typical flexuous bacilli observed in vivo on decaying alder leaves.

Fig. 13 Typical cocci observed in vivo on decaying alder leaves.

Fig. 14 Typical prosthecate bacteria observed in vivo on decaying alder leaves.

Fig. 15 Typical s-shaped bacteria observed in vivo on decaying alder leaves.

Fig. 16 Typical chains of flexuous bacilli separated by "discs" observed in vivo on decaying alder leaves.

Fig. 17 Typical brick-shaped bacilli observed in vivo on decaying alder leaves.

Fig. 18 Analysis of variance between numbers of bacteria/cm² on decaying alder leaves in experimental run number 2.

Fig. 19 Analysis of variance between number of bacteria /cm² on degrading alder leaves in experimental run number 5.

Fig. 20 Analysis of variance between number of bacteria/cm² on alder leaf discs incubated in vitro with river water in a closed system continuous flow fermentor.

Fig. 21 Change in number of bacteria/cm² of alder leaf during run number 2.

Fig. 22 Change in number of bacteria/cm² of alder leaf during run number 5.

Fig. 23 Change in number of bacteria/cm² of alder leaf discs incubated *in vitro* with river water in a closed continuous flow fermentor system.

Fig. 24 Negatively stained TEM preparation of isolate number 151 to show flagellar arrangement and insertion.

Figs. 25-39 Fatty acid profiles of isolates selected for further investigation.

Fig. 40 *Xanthomonas* isolate after 5 days colonisation of sterile alder leaf discs.

Fig. 41 *Erwinia* isolate after 5 days colonisation of sterile alder leaf discs.

Fig. 42 *Janthinobacterium* isolate after 5 days colonisation of sterile alder leaf discs.

Fig. 43 *Bacillus* isolate after 5 days colonisation of sterile alder leaf discs.

Fig. 44 *Ps. testosteroni* after 5 days colonisation of sterile alder leaf discs.

Fig. 45 *Rhodococcus* after 5 days colonisation of sterile alder leaf discs.

Fig. 46 Initial colonisation of alder leaves by microorganisms *in vitro* by a known mixture of microorganisms.

Fig. 47 Diversification and proliferation of microorganisms colonising alder leaves in vitro.

Fig. 48 Diversification and reduction of microorganisms colonising alder leaves in vitro.

Fig. 49 Stabilisation of microorganisms colonising alder leaves in vitro.

Fig. 50 Change in total number of bacteria, cultured on NA, colonising sterile alder leaf discs in vitro.

Fig. 51-54 Change in numbers of individual genera, as a percentage of the total number of colonies cultured on NA, colonising alder leaves in vitro.

Fig. 55 Bacterial burrow hole and erosion pit formation in decaying alder leaves.

Fig. 56 Burrow holes in alder leaves incubated in vitro with a known mixture of microorganisms.

Fig. 57 Organisms with spirochaete-like morphology observed on the surfaces of decaying alder leaves.

Fig. 58 Organism of spirochaete morphology on degrading alder leaf stained with acridine orange.

LIST OF TABLES

Table 1: Comparison of rate of processing of a variety of leaf species.

Table 2: Bacterial genera isolated from two Canadian rivers by random selection and identification of colonies grown on solid media.

Table 3: Distribution of pectin-degrading enzymes in some bacteria.

Table 4: Properties of some microbial endo-lyases (Fogarty and Kelly, 1983).

Table 5: Cellulose degrading bacteria.

Table 6: D-xylanase producing bacteria.

Table 7: Selective media, length of incubation time and method by which organisms removed from decaying alder leaves *in vitro* were enumerated.

Table 8: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 1.

Table 9: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 2.

Table 10: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 3.

Table 11: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 4.

Table 12: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 5.

Table 13: Average water temperatures during incubation periods of alder leaves in the River Bourne.

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA.

Table 15: Morphological and biochemical characteristics obtained by enrichment of isolates from colonised alder leaves with homogenised alder leaves in a mineral medium. Washings were cultured on selective agars.

Table 16: Morphological and biochemical characteristics of organisms on degrading alder leaves enriched in mineral medium with cellophane discs or wicks. The suspensions of organisms subsequently removed from the cellophane were cultured on PSS with microaerophilic incubation.

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium.

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves

onto TSBA and either incubating media with leaves for the full incubation period or removal of leaves after 24 hours and further incubation of the imprinted agar.

Table 19: Morphological and biochemical characteristics of isolates obtained by enrichment in a mineral medium containing dog fur of a suspension of isolates, obtained from colonised alder leaves which had been dried and then rehydrated in water. Washings and dog fur were plated onto chitin agar, oatmeal agar and damp filter paper after a 2-6 week enrichment.

Table 20: Morphological characteristics of streptomycete-like isolates.

Table 21: Identifications of isolates.

Table 22: Bacterial isolates selected for further work.

Table 23: Growth of isolates at 4°C.

Table 24: Dimensions, arrangement and distinguishing characteristics of some isolates selected for further work after colonisation of sterile alder leaf discs as observed under SEM.

Table 25: Polymer content of dried leaf cell walls.

Table 26: Growth and liquefaction of sodium polypectate gel by isolates.

Table 27: Breakdown of sodium polypectate in cup plate assays using supernatants from pectinase inductions.

Table 28: Activity of pectate lyases induced by 18h culture of isolates in pectate lyase induction medium.

Table 29: Breakdown of xylan in xylanase assay plates by isolates.

Table 30: Action of Streptopmyces spp on RBB dyed cellophane, assayed visually and on CMC, after induction with solka floc, assayed by measurement of reducing sugars.

Table 31a: Sums of dry weights of leaves and mean values thereof for use in one way analysis of variance.

Table 31b: Differences between mean dry weights of alder leaves from an uninoculated control and from leaves incubated with microorganisms after 24 days and significance of difference as calculated by one way analysis of variance and Tukey's HSD.

Table 32: Division of isolates obtained from degrading alder leaves (Harris, present study) and from river water, oak and pignut hickory (Suberkropp and Klug, 1976) into classes according to divisions in Bergey's Manual of Systematic Bacteriology vols. 1 and 2.

APPENDIX TABLES

Table 1: Individual counts of bacteria/cm² colonising alder leaves incubated in the River Bourne during run number 2.

Table 2: Individual counts of bacteria/cm² colonising alder leaves incubated in the River Bourne during run number 5.

Table 3: Individual counts of bacteria/cm² colonising sterile alder leaf discs incubated *in vitro* with river water.

Table 4: Dry weight in grams of alder leaves after 24 days incubation with microorganisms. Starting weight = 1 g.

INTRODUCTION

The importance of recycling of nutrients in heterotrophic freshwater streams was recognised as early as the 1920's (Coffman et. al., 1971). A heterotrophic ecosystem has been defined by Boling et. al. (1975) as one in which community respiration exceeds photosynthesis. Allochthonous litter, of which leaves are an important component, accounts for 66 to 99% of the energy input into heterotrophic streams (Kaushik and Hynes, 1971; Fisher and Likens 1973). An autumn pulse of litter is experienced by rivers in decidious forests (Petersen and Cummins, 1974).

The structure and dynamics of stream ecosystems has been studied by a variety of workers, the majority of whom have worked on leaves.

Upon entry into the water a leaf undergoes a period of abiotic leaching of soluble low molecular weight compounds. The length of time for this leaching was considered to be 24 hours by Petersen and Cummins (1974), 3 to 4 days by Kaushik and Hynes (1971), 1 week by Reice (1974) and 2 weeks by Suberkropp et. al. (1976). Triska (1970) also suggested that leaching occurred far more rapidly from thinner leaves. During leaching the litter begins to become colonised by a variety of organisms.

Kaushik and Hynes (1971) compared weight losses of elm (<u>Ulmus americana</u> L.), Maple (<u>Acer saccharum</u> Marsh), oak (<u>Quercus alba</u> L.), beech (<u>Fagus grandifolia</u> Ehrh.) and alder

(Alnus rugosa Du Roi) discs in the Nith and Speed rivers in Wellington County, Ontario, Canada during winter through summer. Elm and maple were found to lose weight much faster than did alder, oak and beech. These workers concluded that temperature, water quality and leaf composition were the most important factors controlling the breakdown of allochthonous litter.

Cummins (1974) in a review, described the rate of decomposition of leaf litter as being temperature dependant. Petersen and Cummins (1974) calculated the biological half life (T_{50}), 80% and 90% stages of decay of a variety of leaf species incubated in Augusta Creek, Barry and Kalamazoo Counties, Michigan USA. Their study enabled them to classify leaf species as slow, medium or fast degraders as shown in Table 1.

Group I	"fast"	Cornus amomum
		Fraxinus americana
Group II	"medium"	Carya glabra
		Salix lucida
		Decodon verticillatus
Group III	"slow"	Quercus alba
		Populus tremuloides

Table 1: Comparison of rate of processing of a variety of leaf species from Petersen and Cummins (1974).

Chamier (1980) working on the decomposition of alder (Alnus glutinosa L.) and oak (Quercus robur L.) in the River

Bourne, Surrey, England found that alder leaves were skeletonised within 10 to 12 weeks and oak after 25 weeks at winter temperatures. In comparing the chemical composition of the two leaf species Chamier suggested that the high lignin content of oak leaves could account for the slower processing. Triska et. al. (1975) calculated a 40% lignin content of oak leaves and 9,5% for alder. Oak leaf tannins have an inhibitory effect on fungal growth (Witkamp, 1963) and this could be another reason for slow degradation of oak. Overall Chamier concluded that geographical location, physico-chemical quality of the water and source of plant material were the three major factors which influenced microbial colonisation of leaves.

A rapid weight loss during the first 6 days incubation was reported for flowering dogwood (*Cornus florida* L.), soft rush (*Juncus effusus* L.) and yellow birch (*Betula lutea* Michx. f.) in the Contrary and Freshwater Creek arms of Lake Anna, Louisa County, Virginia, USA by Carpenter et. al. (1983). Over a further 20 week incubation from June through October dogwood and birch leaves exhibited the greatest changes in dry weight. These authors concluded that the chemical composition of a leaf species is the major factor governing its rate of breakdown, thus supporting Chamier's findings.

Tam et. al (1983) demonstrated a 60% reduction in decomposition of Maple (*Acer saccharum*) incubated *in vitro* at 20°C and 0°C. Witkamp (1963) working on mixtures of white oak (*Quercus alba* L.), American beech (*fagus*

grandifolia Ehrh), red mulberry (Morus rubra L.), sugar maple (Acer saccharum Marsh.) and shumard oak (Quercus shumardii Buckley) in a forest also concluded that temperature, pH and substrate composition were important in controlling rate of breakdown. He also recorded high microbial densities on mulberry leaves and suggested that this could be due to the higher percentage of nitrogenous tissue (C/N = 14) in mulberry when compared to oak (C/N = 36).

There is some evidence that availability of phosphate and nitrate in the environment influences the rate of decomposition. Bell et. al. (1982) concluded from manipulation of phosphate and nitrate levels in chemostat cultures of river water bacteria that nitrogen had a greater influence on decay rate than did phosphate.

Kaushik and Hynes (1971) however demonstrated an increase in mineralisation of elm (Ulmus americana L.) leaves incubated in vitro after enrichment with phosphate. Fenchel and Barker Jorgensen (1977) concluded similarly that the influence of phosphate on the microbial community might ultimately limit its size.

Before the recognition of the importance of microorganisms as primary decomposers of allochthonous litter it was generally accepted that animals accounted for most of the decomposition (Fenchel and Harrison, 1976). There is some evidence which suggests that animals are not able to assimilate the greater part of ingested litter. Hargrave

(1970) estimated that only 5% of elm (Ulmus sp.) leaves ingested by Hyallela azteca was assimilated. Hudson (1972) working on woodland detritus feeders showed that 60 to 90% of the detritus eaten was returned to the soil as faecal pellets. Similar results were also obtained by Bärlocher and Kendrick (1975) who found that Gammarus pseudolimnaeus assimilated 17 to 19% and 10% respectively of maple (Acer saccharum Marsh) and elm (Ulmus sp.) leaves ingested. However, Nilsson (1974) recorded a 30 to 40% assimilation of alder (Alnus sp.) leaves by Gammarus pulex. Chamier and Willoughby (1986) also demonstrated the presence of endogenous cellulase, chitinase and β1-3 glucanase in the guts of Gammarus pulex (L.).

Bärlocher and Kendrick (1973) suggested that animals are dependant upon the earlier activity of fungi and bacteria to gain access to the energy present in the leaf litter.

Kaushik and Hynes (1971) demonstrated that Gammarus sp. exhibited a preference for elm when offered elm, maple, oak, beech and alder leaf discs but when offered uncolonised or microbially conditioned elm leaves it fed preferentially on the latter. Iversen (1973) also found that Sericostoma personatum displayed a preference for microbially conditioned leaves.

Baker and Bradnam (1976) in an attempt to quantify the contribution made by bacteria in the diet of Simulium sp in the River Frome, Dorset, UK concluded that while Simulium fed preferentially on bacteria, the bacteria accounted for

less than 50% of its total energy intake. These results were compared with those of Fredeen (1964) who reared Simulium in a laboratory system entirely on a diet of bacteria. Baker and Bradnam criticise these results as being unrepresentative of the qualitative value of microorganisms when compared with organic matter, but suggested that while bacteria might well be capable of supporting growth of Simulium sp in a laboratory system (provided sufficient quantities of bacteria are offered) their own results suggest that bacteria were not present in the River Frome in sufficient quantity to support growth of Simulium. They further calculate that at least 17% of the suspended solids in the River Frome consist of easily assimilable proteins and fats and suggested that such non-living detrital material accounts for more than 50% of the Simulium energy.

Reice (1974) investigated the affect of sample pack size of white ash (Fraxinus americana) on decay rate. He found that decay rate decreased with increasing pack size during summer but that it increased with increasing pack size during winter. He thought that the elevated decay rates in smaller leaf packs could be due to physical or microbial processes, but that pack size, selective feeding or differential mortality could account for the reverse situation observed during winter. He suggested that the difference arose because different levels of microbial activity in the packs are mediated by physical factors and that differential regimes, variable accessibility and also possibly protection

from the elements could affect colonisation.

King et. al. (1987) studied the dynamics of natural and artificial leaf packs in the Langrivier, Cape, South Africa. They demonstrated that packs consisting exclusively of leaves seldom arose naturally and that in practice packs consisted of twigs and leaves at all stages of decay. They suggested that naturally occurring leaf packs would therefore attract a greater range of detritivores. These authors suggested that differences between detritivores colonising natural and artificial leaf packs were partly due to positioning of the leaf packs; ie artificial packs usually being placed in areas where they would be totally submerged and thus in a "pool", and natural assemblages of leaves more often occurring at or near the air/water interface in "riffles". They further suggest that the lack of contact of naturally occurring packs with the benthos compared to the closer contact of artificial packs with the benthos could create conditions which would encourage the formation of a discrete microbial community in naturally occurring leaf packs rather than an extension of the benthic community experienced in artificial leaf packs. They conclude however that decomposition of detritus in artificial leaf packs insofar as stream detritivores is concerned provides much useful information, but that these packs do not simulate the natural environment.

Recent papers have shown that there is a variety of microorganisms on decaying litter and that they colonise in a specific and recognisable pattern. Thus far fungi have

been studied more extensively than bacteria and evidence has shown that the aquatic Hyphomycetes are the predominant fungi encountered on decaying leaves in freshwater. The relative importance of bacteria and fungi in leaf decay is difficult to define, but since at this stage there have been more detailed studies of the fungi, it may be that the importance of bacteria has been somewhat neglected.

Kaushik and Hynes (1971) characterised the fungi present on the surfaces of elm, maple, oak, beech and alder leaves in freshwater. They identified ten species of geofungi which were regularly observed on leaf surfaces and also in the stream. Spores of the aquatic Hyphomycetes Tricladium, Tetracladium and Alatospora were also observed.

An extensive study of the fungal flora on ash (Fraxinus pensylvanica Marsh.), maple and oak in the Speed River, Ontario, Canada was conducted by Bärlocher and Kendrick (1974). Their conclusions show that aquatic Hyphomycetes were the dominant flora observed sporulating on leaves underwater. Many of the geofungi observed by these authors had previously been observed by Kaushik and Hynes (1971). Bärlocher and Kendrick also noted that many of the geofungi present on leaf surfaces in the stream were present on the leaf surfaces prior to abscision. Aquatic Hyphomycetes were observed to be the dominant flora on leaf surfaces during the colder months, although similar proportions of aquatic Hyphomycetes and geofungi were observed during the warmer months and the authors therefore concluded that geofungi were at least as important as the aquatic Hyphomycetes in

degrading leaf litter during the warmer seasons. Bärlocher and Kendrick thought that the tetraradiate spore shape of the aquatic Hyphomycetes and their ability to sporulate underwater accounted for their success in leaf litter degradation.

Suberkropp and Klug (1974) working on oak and pignut hickory (Carya glabra) in Augusta Creek demonstrated a succession of aquatic Hyphomycetes by direct observation of the leaf and after allowing the fungi to grow on agar media. In the same study bacteria on the leaf surfaces were investigated by plating of leaf homogenates onto agar media and subsequent identification of representative colonies, but they were unable to demonstrate a similar colonisation pattern.

The same authors (1976) studied bacteria and fungi associated with decaying oak and pignut hickory leaves in Augusta Creek and they concluded that fungi dominated the microbial biomass in at least the first 18 weeks of leaf litter breakdown. The dominant fungi were Flagellospora curvula, Lemonniera aquatica, Alatospora acuminata, Tetracladium marchalianum, Anguillospora sp and Clavariopsis aquatica. They reported preliminary experiments which indicated that the dominant fungi, because they produced arrays of pectinases and cellulases, were more invasive than bacteria during the early stages of leaf degradation.

Representative bacterial colonies from peptone-yeast-extract-glucose agar were also identified and the major

bacterial genera isolated were Flavobacterium, Flexibacter, Pseudomonas, Acinetobacter, Achromobacter and Chromobacterium. Serratia, Alcaligenes, Bacillus, Cytophaga, Sporocytophaya and Arthrobacter were also isolated, but less frequently.

Chamier (1980) working on alder and oak leaves in the River Bourne demonstrated distinct and different patterns of colonisation of the two leaf species by aquatic Hyphomycetes. The dominant aquatic Hyphomycetes were Tetrachaetum elegans, Anguillospora longissima and Mycocentrospora angulata. Species encountered less often were Lemonniera aquatica and Alatospora acuminata on oak and L. aquatica and Lemonniera terrestris on alder. Chamier also enumerated bacteria removed from the surfaces of decaying leaves plated onto Nutrient agar and Pectin agar. The increase in pectolytic bacteria was found to mirror the increase in aquatic Hyphomycete spore numbers.

Early work on forest litter by Ivarson and Sowden (1959) reported rates of microbial breakdown to remain unchanged after antibiotic treatment. They concluded that bacteria and fungi were equally efficient in breakdown of leaf litter.

Tam et. al. (1983) compared the effect of adding an antifungal or antibacterial antibiotic to maple leaves in freshwater stream sediment from Faradale, Ontario, Canada. The loss of total carbohydrate during a 35 day incubation period was greater when using an antifungal antibiotic while

the greatest loss was achieved when neither antifungal nor antibacterial antibiotic was used.

Bacteria are of fundamental importance in recycling carbon, nitrogen and phosphorous within an ecosystem. Fenchel and Barker Jorgensen (1977) considered that they are important primary decomposers for the following reasons:-

1. They can utilize dissolved organic substrates at low concentrations.

2. They can assimilate dissolved organic nutrients and can also decompose nutrient poor substrates. These properties are due to their large surface to volume ratio.

3. Bacteria have enzyme systems which can attack structural plant compounds.

4. Many bacteria have efficient modes of anaerobic respiration.

Scanning electron microscope (SEM) studies of bacterial flora on degrading planting material in aqueous and aquatic environments is limited. Brock (1987) however comments favourably on its use as a tool with which microorganisms can be studied in their natural environment. It is hoped that this work will make a contribution in this area.

Federle and Vestal (1980 & 1982) studied the colonisation of sedge (Carex) leaf litter in Lake Toolik, Alaska using SEM.

This study was conducted during summer since Lake Toolik is covered by a layer of ice from October to June.

Microbial forms were not observed on the dried sedge litter prior to incubation in the water. Debris accumulated on the leaf surfaces within 12 hours of incubation but no microbial forms were observed. Scattered bacteria-like forms, some invading stomata, and small microcolonies were observed after 3 days incubation as well as some very isolated filamentous forms.

The variety and density of the colonising organisms had increased after 4 days and rod-shaped bacteria-like forms were most frequently observed.

Large filaments observed under low magnification (30 x) were highly prevalent after 7 days incubation but appeared fragmented and deteriorated after 13 days incubation. The authors were unable to determine whether these filaments were of fungal or algal origin, but suggested that the increased rate of weight loss from the leaves recorded during colonisation by these filaments indicated that they may be of fungal origin.

Actinomycete-like forms and bacteria-like forms enmeshed in slime were also observed after 7 days incubation, the latter becoming increasingly conspicuous and after 13 days incubation the entire leaf surface was covered by slime. The occurrence of these slime-covered bacteria-like forms then declined and slime was not observed on leaves which had been incubated for 28 days.

Diatoms were first observed, in isolation, after 17 days incubation and thereafter tended to become more abundant and more diverse. Similar flora consisting of diatoms and bacteria-like forms were observed on leaf litter incubated for 28 days and for one year.

Federle and Vestal thus defined colonisation as a four-stage process:-

1. initial colonisation
2. explosive growth
3. decline
4. stabilisation and continued succession.

These conclusions were further supported by measurements of carbon, nitrogen, phosphorous and protein content of the leaf litter and by analysis of ATP in microorganisms.

Federle and Vestal concluded that bacteria initially dominate the colonising flora. These findings do not support earlier reports by Suberkropp and Klug (1974) who studied the colonisation of leaf litter in Augusta Creek using SEM. They demonstrated a predominance of aquatic Hyphomycetes during the initial stages of decay although viewing of bacteria might have been limited by the use of low magnification.

Morrison et. al. (1977) investigated the colonisation of oak (Quercus virginiana) and slash pine (Pinus elliottii) incubated in Apalachicola Bay, Florida, U.S.A. during

summer. They measured phospholipid and neutral lipid synthesis and enzymatic and respiratory activities and compared the activities with active biomass as measured by ATP and also SEM observations. They recorded high ratios of muramic acid to ATP during the first two weeks of the six week incubation, indicating that bacteria were the initial colonisers. SEM observation of both oak and slash pine supported these findings. These findings do not support those of Suberkropp and Klug (1974) although they are not strictly speaking comparable because Morrison et. al.'s study was conducted in salt water and Suberkropp and Klug's study in freshwater.

Paerl (1969) observed filamentous bacteria, true bacteria and fungi on detritus in Lake Tahoe, east California, west Nevada, U.S.A. Bacteria observed on detritus removed from the top 20 m of the water column were always closely associated with fungal mycelia and detrital surfaces, and were often observed surrounded by a web-like network which was not observed surrounding free-floating bacteria. They also observed fungi growing into the surfaces of detritus.

Suberkropp and Klug suggested that fungi were important during the early stages of decay because they were able to penetrate the leaf surface. They thought that bacteria were initially limited to the surface of the leaf or to entry via the stomata only gaining better access once the leaf cuticle was removed.

Holt and Jones (1978) and Mouzouras et. al. (1987) however have demonstrated the presence of bacteria which are able to burrow and tunnel into wood. Holt and Jones (1978) demonstrated that bacteria were responsible for cavity formation in the secondary cell wall of delignified beech (Fagus sylvatica) and Scots pine (Pinus sylvestris) in vitro. They also demonstrated that bacteria were able to degrade lignified beech and Scots pine in anaerobic aquatic habitats (Holt and Jones, 1983) and noted that rod-shaped bacteria created erosion troughs along a specific axis of the wood cells.

Holt et. al. (1979) demonstrated that bacteria colonised four different species of wood block in marine anaerobic mud in a two-stage pattern. Colonisation commenced with establishment of one or more different bacteria including rod-shaped organisms, cocci, spirochaetes and actinomycetes. Many of these organisms produced mucilage which the authors consider may assist in attachment or in direction and concentration of extracellular enzyme. Breakdown of at least one wood species appeared to be confined to rod-shaped bacteria, which were often observed surrounded by small troughs of decay. These pit areas became severely eroded after prolonged exposure of the wood.

Mouzouras et. al. (1983) also described the pattern of microbial colonisation of four different species of wood incubated in 600 mm of seawater for 12 weeks. They found that coccoid, prosthecate and rod-shaped organisms initiated colonisation and that they were often associated with

polysaccharide. Rod-shaped and prosthecate bacteria were the most abundant microorganisms. The prosthecate organisms then decreased in numbers and actinomycete bacteria colonised. Rod-shaped organisms were still in abundance, thinner ones being more common than broader ones. The surfaces of the wood blocks were covered by a layer of microalgae after 12 weeks incubation and cocci and actinomycetes were the dominant bacteria. This pattern of colonisation was common to the four different species of wood tested.

Mouzouras et. al. also described the action of burrowing and tunnelling bacteria observed in their study. The ability to burrow or tunnel appeared to be manifested in rod-shaped bacteria with rounded ends. The organsims attached either polarly or at an angle to the substrate, producing little or no mucilage or else sufficient to cover the entire organism. The organisms which produced little or no mucilage penetrated the S_3 layer of the wood, leaving a slightly enlarged, diamond-shaped hole. It appeared that burrowing commenced inside the mucilage cover for those organisms which produced much mucilage, and the resulting bore holes only became visible once the organism had penetrated the S_2 layer.

Evidence of burrowing was also obtained in this study and is presented.

Aquatic hyphomycetes and bacteria which degrade allochthonous litter are generally not present on the leaf

surface at time of senescence but are river or runoff associated (Bärlocher and Kendrick, 1974; Suberkropp and Klug, 1976; Goulder, 1980; Nutall, 1982). The composition of river microorganisms would therefore have an effect on the nature of the bacteria which colonise the detritus. Furthermore, bacteria in temperate rivers are liable to seasonal periodicity in response to changes in physio-chemical character of the river during the different seasons (Goulder, 1986).

Bell et. al. (1980) studied the bacterial populations of two Canadian rivers and identified twelve different genera (Table 2) by randomly picking and identifying colonies from solid media.

Pseudomonas	Cytophaga
Alcaligenes	Flexibacter
Aeromonas	Escherichia
Chromobacterium	Enterobacter
Plesiomonas	Klebsiella
Flavobacterium	Acinetobacter

Table 2: Bacterial genera isolated from two Canadian rivers by random selection and identification of colonies grown on solid media (Bell et. al., 1980)

Representatives of all genera were present in both rivers during summer and winter although frequency of isolation of species fluctuated between the seasons. The bacterial populations from both rivers were almost exclusively

psychrotrophic in nature with growth temperature optima of 26°C.

Several different concentration patterns of bacteria have been described. Summer maxima, winter maxima and no pattern have been shown by both direct enumeration and plate counts (Goulder, 1986). Studies on bacterial heterotrophic activity have, however, demonstrated summer maxima (Goulder, 1986 and references therein).

Bell et. al. (1980) made a detailed study of the physico-chemical nature of the Rivers Meduxnekeag and Dunbar in Canada and related these findings to measurements of bacterial heterotrophic activity. They concluded that temperature had the greatest influence on heterotrophic activity.

Goulder (1986) studied seasonal variation of free-living and particle-bound bacteria in the Rivers Ouse and Derwent, Yorkshire, U.K. He recorded a higher concentration of free-living bacteria during summer in both rivers using direct enumeration and correlated these findings with increased temperature, low upstream treated sewage discharge and a high concentration of phytoplanktonic pigments. Particle-bound bacteria, also directly enumerated, peaked during winter. Goulder considered this to be in response to high discharge rate of treated sewage and large amounts of suspended solids and particulate organic matter. Totals of colony-forming-units on solid media were positively correlated with numbers of particle-bound bacteria also

cultured on solid media which suggested that bacteria cultured on solid media were either particle-bound or another element of the community whose abundance mirrored that of particle- bound bacteria. Goulder did not detect a seasonal pattern in total bacterial concentration because his measurements included free-living and particle-bound bacteria which peaked during different seasons. He did however record an increase in heterotrophic activity during summer which appeared to be due to increased concentration of free-living bacteria and also to summer river conditions (Goulder 1980; 1986).

In order to effect efficient breakdown of plant material a microorganism must be able to break down the plant cell wall. This is assisted by a complex set of enzymes which degrade the major structural polymers which are cellulose, hemicellulose and pectin. Wood (1966) commented that only the enzymes with pectin and cellulose as substrates had been studied to any great extent and this still holds today largely because cellulose is the most abundant polysaccharide in the plant kingdom (Bateman and Basham, 1976) and the destruction of cellulose and pectin is also of major economic importance.

Cell walls are responsible for maintaining the shape and integrity of plants. They are arranged into three distinct regions on the basis of their chemical composition and degree of organisation. These are the pectin-rich middle lamella, the primary cell wall which is composed of cellulose, hemicellulose and pectic polysaccharide and the

secondary wall which is composed largely of cellulose and some hemicellulose (Bateman and Basham, 1976; Darvill et. al., 1980).

The primary cell wall is laid down by undifferentiated cells during growth (Darvill et. al., 1980) and as long as the cell grows this primary wall is composed of 90% polysaccharide and 10% protein (John and Dey, 1986). Secondary cell walls and hydroxyproline-rich protein are deposited once the cells have ceased growth and become differentiated (Bateman and Basham, 1976 and references therein).

The ratio between major cell wall constituents varies depending on plant species and location. Generally cell walls contain 15-45% pectic polysaccharides, 15-25% hemicellulose, 30-60% α-cellulose and 10-15% glycoproteins (Selvendran, 1983). McNeil et. al. (in John and Dey, 1986) working on suspended sycamore cells found the cell walls to consist of 34% pectic polysaccharide, 24% hemicellulose, 23% cellulose and 19% glycoprotein.

The pectic substances are composed primarily of α 1-4 linked D-galacturonopyranose interspersed with 1,2 linked rhamnopyranose (Darvill et. al, 1980). Pectic polysaccharides occur in covalent association with galacturonosyl-containing polysaccharides (Darvill et. al., 1980).

Hemicellulose is considered to be made up of xyloglucans and glucuroarabinoxylans (Bateman and Basham, 1976; John and

Dey, 1986). Functionally they link the pectic and cellulosic fractions (Northcote, 1972 and Bauer et. al. 1973 in Bateman and Basham, 1976).

Cellulose is the major structural component of plant cell walls. Chemically, cellulose consists of long, linear chains of β1-4 linked glucosyl residues (Darvill et. al., 1980). The chain is stabilised by hydrogen bonding between adjacent glucose residues. Cellulose fibrils range from 3,5 to 35 nm in diameter and are randomly arranged in the primary cell wall, but occur in parallel lamellae in the secondary cell wall (Bateman and Basham, 1976). Crystalline and amorphous regions occur within the cellulose fibrils in primary and secondary walls.

There is evidence to suggest that cell wall degrading enzymes are produced in a specific pattern (Wood, 1966 and references therein; Bateman and Basham, 1976). Wood (1966) states that secretion of leaf degrading enzymes is almost always induced by the substrate and that cellulases would therefore be unlikely to be produced before the cellulosic areas of the leaf were exposed by the action of pectic enzymes (maceration).

Codner (1971) demonstrated that the cellulases produced by cellulolytic organisms only become active once the tissue had been macerated, confirming earlier reports on the inducible nature of these enzymes. However, Ianotti et. al. (1973) reported presence of a constitutive component of the cellulase system of <u>Ruminococcus albus</u>. They recorded

elevated levels of cellulase production in cultures grown with cellulose when compared with those offered cellobiose. They also observed rapid growth of glucose-adapted cells in a cellulose medium which tended to support their earlier observations. They could not, however, say whether the increased amounts of enzyme were produced as a result of increased constitutive action in response to the added cellulose.

Pectinases are produced by microorganisms and plants and in bacteria they are often associated with pathogenicity (Smith, 1958; Wood, 1966; Starr and Nasuno, 1967; Nasuno and Starr, 1967; Codner, 1971; Rexova-Benkova and Markovic, 1976; Obi, 1981; Chamier and Dixon 1982a & b; Fogarty and Kelly, 1983; Bashan et. al., 1988; Chamier, 1985). Pectinases are classified as either pectinesterases which de-esterify pectin or as depolymerizing enzymes which catalyze the cleavage of α 1-4 bonds of the polymer. Depolymerizing enzymes are further divided into three groups as follows:

1. Whether pectin, pectic acid or oligo-D-galacturonate is the preferred substrate.

2. Whether they act by transeliminative cleavage or by hydrolysis.

3. Whether degradation is random (endo) or from the ends (exo).

Pectinases vary in molecular weight and have been reported as low as 23 000 and as high as 360 000 (Fogarty and Kelly, 1983).

Table 3 shows the range of pectinases produced by some bacteria.

Source	PE	PG	PGL	PMG	PMGL	OG	OGL
Bacillus sp.			+			+	
Bacillus sp. No. RK9			+				
Bacillus subtilis			+				
Bacillus polymyxa			+				
Bacillus pumilus			+				
Bacillus sphaericus			+				
Bacillus stearothermophilus			+				
Erwinia aroideae		+	+		+		+
Erwinia carotovora		+	+				+
Pseudomonas sp.			+				+
Pseudomonas fluorescens			+				
Pseudomonas marginalis	+	+	+				
Xanthomonas sp.	+		+				
Xanthomonas campestris	+		+				
Xanthomonas cyanopsidis			+				
Clostridium multifermentans	+		+				
Clostridium aurantibutyricum	+		+				

Source	PE	PG	PGL	PMG	PMGL	OG	OGL
Clostridium felsineum		+	+				
Cytophaga johnsonii			+				
Cytophaga deprimata			+				
Cytophaga albogilva			+				
Streptomyces nitro-sporeus			+				

Table 3: Distribution of pectin-degrading enzymes in same bacteria (Fogarty and Kelly, 1983).

PE = pectin esterase

PG = polygalacturonase

PGL = polygalacturonate lyase

PMG = polymethylgalacturonase

PMGL = polymethylgalacturonate lyase

OG = oligogalacturonase

OGL = oligogalacturonate lyase

Pectinesterases are produced by higher plants, moulds, yeasts and bacteria although knowledge concerning these enzymes is limited. Pectinesterases display a high specificity for the methyl ester of pectic acid. Pectin is deesterified in a linear fashion with the release of free carboxyl groups (Fogarty and Kelly, 1983).

Pectic hydrolases are divided into three groups which are endopolygalacturonases, exopolygalacturonases and oligogalacturonases. Some authors have also suggested that

a fourth pectic hydrolase, endopolymethylgalacturonase, exists (Fogarty and Kelly, 1983). Endo- and exopolygalacturonases are either cell-bound or extracellular enzymes which attack pectic acid. Endopolygalacturonases are produced by fungi, bacteria, yeasts and higher plants, and exopolygalacturonases by fungi and bacteria. Oligogalacturonases are cell-bound enzymes produced by bacteria and fungi (Fogarty and Kelly, 1983).

Pectin lyases are divided into four groups which are endoplolygalacturonate lyase, exopolygalacturonate lyase, oligogalacturonate lyase and endomethylgalacturonate lyase. They are produced by a variety of fungi and bacteria, although endomethylgalacturonate lyase production by bacteria is rare (Fogarty and Kelly, 1983).

Pectin lyases can be either cell-bound or extracellular with the exception that oligogalacturonate lyases are always cell-bound (Fogarty and Kelly, 1983).

The majority of pectin lyases have an absolute requirement for calcium ions for their activity. It has been suggested that the calcium ions chelate pairs of oligogalacturonides in the substrate and that it is on these connecting salt bridges that the enzyme acts (Fogarty and Kelly, 1983).

Many of the organisms in this study showed lyase activity. Some properties of pectin lyases produced by bacteria are shown in table 4.

Organism	pH optimum	Molecular Weight
Pectin Lyases		
Erwinia aroideae	8,1	30 000
Polygalacturonate Lyases		
Bacillus circulans I	10,0	70 000
II	9,5	18 000
Bacillus subtilis	8,5	33 000
Bacillus subtilis	8,0	49 000
Bacillus sp. RK9	10,0	
Bacillus stearothermophilus	9,0	24 000
Erwinia aroideae	9,1	37 000
Erwinia caratovora	8,5	
Pseudomonas fluorescens	9,4	42 300
Streptomyces nitrosporeus	10,0	41 000

Table 4: Properties of some microbial endo-lyases (Fogarty and Kelly, 1983)

Cellulases are produced by a wide variety of fungi and bacteria. Evidence shows that different cellulases act together in order to effect total cellulose breakdown (Enari, 1983). It is thought that the crystalline regions of cellulose are less susceptible to microbial breakdown than are the amorphous regions and that the relative proportions of crystalline to amorphous cellulose in any

particular sample therefore has an effect on the ease with which a substrate is broken down (Codner, 1971).

Myxobacteria, actinomycetes and true bacteria have been reported to produce cellulase. Some cellulase producing bacteria are shown in table 5.

Myxobacteriales	Sporocytophaga
	Cytophaga
Actinomycetales	Micromonospora
	Streptomyces
	Thermoactinomyces
	Thermomonospora
	Thermopolyspora
Eubacteriales	Bacillus
	Cellulomonas
	Pseudomonas (Cellvibrio)
	Clostridium
	Bacteriodes
	Ruminococcus
	Erwinia

Table 5: Cellulose degrading bacteria (Enari, 1983; Andro, 1984)

Work on bacterial cellulases had centred largely on a qualitative rather than on a quantitative level and little is known of the mechanism of action. Cellulose breakdown is accomplished by the synergistic and possibly sequential

action of endo-β-glucanases, exo-β-glucanases and β-glucosidases (Enari, 1983).

Pettipher and Latham (1979) reported production of an array of cell wall degrading enzymes by Ruminococcus flavefaciens. They demonstrated cellulases which converted carboxoymethylcellulose to cellobiose or cellotriose. These authors thought that the most active enzymes produced by R.flavefaciens were exo 1,4 β-glucosidases which preferentially hydrolysed the second and third β-glycosidic bonds from the end of the polymers. This hypothesis was supported by the rapid release of reducing sugars but only slight decrease in viscosity of the substrate.

Hemicellulases are those enzymes which specifically degrade hemicelluloses and they include L-arabinanases, D-galactanases, D-mannanases and D-xylanases (Dekker and Richards, 1976). A survey of D-xylanases produced by some bacteria isolated in the present study was undertaken.

There are two types of xylanase recognised and these are 1-3 β-D-xylanase and 1-4 β-D-xylanase (Dekker and Richards, 1976). The latter enzyme is produced by many terrestrial bacteria as shown in table 6.

Actinomyces

Bacillus

Cellvibrio

Microbispora

Micromonospora

Nocardia

Sporocytophaga

Streptomyces

Table 6: D-xylanase producing bacteria (Dekker and Richards, 1976)

D-xylanases are produced inductively or constitutively by bacteria (Dekker and Richards, 1976). Work on D-xylanases produced by *Bacillus subtilis* and *Streptomyces xylophagus* has shown that they are mainly of the endo type and that both degraded D-xylan to D-xylose on prolonged incubation (Dekker and Richards, 1976).

AIMS

Many different micro- and macroorganisms are associated with decomposition of allochthonous leaf litter in freshwater streams. Early work concentrated on the role played by stream invertebrates while more recently work has focussed mainly on fungi, more particularly the aquatic Hyphomycetes, and to lesser extent, on bacteria. It has been demonstrated that aquatic Hyphomycetes colonise degrading leaf litter in a specific and recognisable pattern and that they actively produce plant cell wall degrading enzymes. Furthermore they

are well adapted to an aqueous environment by virtue of their ability to sporulate underwater.

Bacteria are always associated with degrading allochthonous litter, but their role in leaf decomposition remains unresolved. The aims of this study were therefore threefold; firstly to investigate the colonisation of leaf litter by bacteria; secondly to isolate and characterise the bacteria present on the degrading leaves and thirdly to survey production of cell wall degrading enzymes by a representative sample of bacteria in order that their role in leaf decay might be better understood.

MATERIALS AND METHODS

1. THE SITE

The field work was carried out in the River Bourne (Grid ref. OS sheet no. 170 SU987678).

Earlier work by Chamier (1980) found oxygen saturation to be approximately 90%, nitrate levels of at least 0,35mg/l, phosphate to range from being undetectable to 0,08mg/l and pH to be neutral to slightly alkaline at this site.

2. LEAVES AND LEAF PACKS

Alder (*Alnus glutinosa*) leaves were selected for this study since it is a common species found along the banks of the River Bourne (Chamier, 1980).

Abscised alder leaves were collected daily from the surface of a nylon net suspended 1 m above the ground in a stand of alders in the grounds of the Department of Biology, Royal Holloway and Bedford New College. The leaves were not allowed to fall onto the ground to avoid leaching of minerals and attack by soil microflora and fauna. Leaves were airdried on paper towels at room temperature (19-22°C) and subsequently stored in well ventilated plastic sacks at room temperature until required

3. LEAF PACK CONSTRUCTION

Sheets of 3mm diamond mesh netlon, 280 mm x 190 mm were

doubled over widthways and stitched with nylon fishing line along the long and one short side to form leaf pack bags. Five dried leaves (approximately 1 g) were placed into each bag which was then stitch-closed on the open side. Packs were anchored to the river bed by a 20-30 cm length of nylon fishing line attached to a masonry brick. Three packs were attached to each brick. Packs were placed approximately 1 m from the river bank in a depth of 10-20 cm of water.

4. COLLECTION AND PROCESSING OF LEAF PACKS

After the appropriate incubation periods leaf packs were removed from the stream and immediately placed into sterile plastic bags. A small amount of river water from the site was added to each bag to ensure a high level of moisture. The bags were then transported to the laboratory for processing, a journey of approximately 10 minutes. Temperature of the stream was recorded each time leaves were sampled.

5. MEDIA, STAINS AND REAGENTS

Media, stains and reagents are listed in the order in which they first appear in the text.

Unless otherwise stated the pH of the medium was adjusted using 1M NaOH and 1M HCl, sterilised by autoclaving at 121°C for 15 minutes and poured into petri dishes.

<u>Peptone Succinic Acid Medium</u> (i)

Hylemon et al., (1973)

In g/l	10,0	peptone
	1,0	succinic acid
	1,0	$(NH_4)_2 SO_4$
	1,0	$MgSO_4.7H_2O$
	0,002	$FeCl_3.6H_2O$
	0,002	$MnSO_4.H_2O$
	7,00	agar

The pH was adjusted to 6,8 with KOH and the medium sterilised.

<u>Oatmeal Agar</u> (ii)

Shirling and Gottlieb (1966)

Trace Salts Solution

In g/l	1,0	$FeSO_4.7H_2O$
	1,0	$MnCl_2.4H_2O$
	1,0	$ZnSO_4.7H_2O$
	20,0	oatmeal
	18,0	agar

20 g oatmeal was steamed in 1000 ml distilled water for 20 minutes and then filtered through cheesecloth. The volume was restored to 1000 ml with distilled water. 1 ml of trace salts was added and the pH adjusted to 7,2 prior to sterilization.

<u>TA Medium</u> (iii)

Keddie et al. (1966)

In g/l	2,5	peptone
	2,5	yeast extract

	20,0	agar

The pH was adjusted to 6,8 and the medium sterilised.

<u>Chitin Agar</u> (iv)

Willoughby (1966)

In g/l	1,7	KCl
	4,1	$Na_2.HPO_4.12H_2O$ (added first and allow to dissolve)
	0,05	$MgSO_4.7H_2O$
	0,2	$CaCO_3$ (added last)
	0,01	$FeSO_4.7H_2O$
	2,5	chitin
	18	agar

The chitin was dissolved in 70 ml 50% (v/v) H_2SO_4 for 90 minutes, filtered through sintered glass and diluted to 15 times volume with 1050 ml distilled water. The washings were centrifuged at 2000 rpm until a pH of 4 or above was reached. The volume was made up to 1000 ml and the remaining compounds added. The pH was adjusted to 7,2 with H_2SO_4 and the medium sterilised.

<u>Pectin Agar</u> (v)

Hankin et al. (1971)

In g/l	2	$(NH_4)_2SO_4$
	4	KH_2PO_4
	6	Na_2HPO_4
	0,2	$MgSO_4$
In µg/l	1	$FeSO_4.7H_2O$
	1	$CaCl_2$

In µg/l	10	H_3BO_3
	10	$MnSO_4$
	70	$ZnSO_4$
	50	$CuSO_4$
	10	MoO_3

The pH was adjusted to 7,4 and then 15 g agar, 5g pectin and 1g yeast extract was added. The medium was sterilised by autoclaving and plates poured. Plates were developed with 1% hexodecyltrimethylammoniumbromide in order to visualise areas of pectin breakdown.

Leaf Homogenate Agar (vi)

Deschamps et al. (1980) with modifications

In g/l	2,6	$(NH_4)_2SO_4$
	1	K_2HPO_4
	0,5	KH_2PO_4
	0,2	$MgSO_4.7H_2O$
	0,01	$CaCl_2$
	0,001	$FeSO_4$
	0,1	yeast extract
	10	leaf homogenate
	18	agar

The pH was adjusted to 7,2 and the medium sterilised.

Paste Medium (vii)

Willoughby et al. (1984) with modifications

In g/100 ml	6	peptone
	0,76	$MgSO_4.7H_2O$
	0,008	KH_2PO_4

In mg/100 ml	2,4	$CaCl_2$
	0,15	$FeSO_4.7H_2O$
	0,15	$MnSO_4$
	0,03	$CuSO_4$
	0,03	$ZnSO_4$

30 ml of the above solution was added to 970 ml distilled water. Th pH was not adjusted. After sterilisation 30 ml aliquots were dispensed into petri dishes and 0,9 g Polycell Regular Wallpaper paste (Polycell, Hertfordshire, England) was added to each. The medium set as a semi-solid.

Yeast-Extract Malt-Extract Agar (viii)

Shirling and Gottlieb (1966)

In g/l	4,0	Bacto-yeast extract
	10,0	Bacto-malt extract
	4,0	Bacto-dextrose
	20,0	Bacto-agar

The medium was adjusted to 7,3 and sterilised.

Tap Water Agar (ix)

Cross, personal communication

In g/l	18	purified agar
		tapwater

The pH was not adjusted prior to sterilisation.

Enrichment Medium (x)

Microorganisms were enriched for in a distilled water and leaf homogenate medium.

Dried alder leaves were ground up in a small quantity of distilled water using a pestle and mortar and made up to 100 ml giving concentrations of material which ranged from 10^{-2} to 10^{-6} g/ml. Each 100 ml aliquot was dispensed into a 500 ml conical flask. The pH of the medium was adjusted to 7,0 with sterile 4% sodium bicarbonate after autoclaving.

Tryptone Soy Broth Agar (xi)

Zucker and Hankin (1970)

In g/l	17	tryptone
	3,0	soy peptone
	2,5	dextrose
	5,0	NaCl
	2,5	K_2HPO_4

The pH was adjusted to 7,3 ml and the medium sterilised.

Dye's Medium C for Acid Production from Carbohydrates by Xanthomonas spp Dye (1962) (xii)

In g/l	0,5	$NH_4H_2PO_4$
	0,5	K_2HPO_4
	0,2	$MgSO_4.7H_2O$
	5,0	NaCl
	1,0	yeast extract
	12	agar
	5	carbon source

0,7 ml of a 1,5% alcoholic solution of bromocresol purple was added to the medium which was then boiled to

dissolve the agar. The pH was adjusted to 7,2 and the medium dispensed into test tubes, sterilised and allowed to set in the slant position.

Tryptone Broth (xiii)

Salle (1948)

In g/l	10	tryptone
	3	meat extract

pH was adjusted to 7,2 and the medium sterilised.

Simmon's Citrate Agar (xiv)

Merck Catalogue

In g/l	1,0	$NH_4H_2PO_4$
	1,0	K_2HPO_4
	5,0	NaCl
	2,0	$Na_3C_6H_5O_7$
	0,2	$MgSO_4.7H_2O$
	0,08	bromothymol blue
	12,5	agar

The agar was dissolved in the medium by boiling and the pH adjusted to 6,9. The medium was dispensed into test tubes, sterilised and allowed to set in the slant position.

Starch Agar (xv)

Ayers, Rupp and Jonson Base (NCPPB notes, 1987).

In g/l	1	$NH_4H_2PO_4$
	0,2	KCl
	0,2	$MgSO_4.7H_2O$
	2,0	soluble starch

	12,0	agar

pH was adjusted to 7,4, the medium sterilised and plates poured. Plates were developed with an iodine solution in order to visualise areas of starch breakdown.

Gelatin Medium (xvi)

Gardner (1969)

In g/l	5,0	NaCl
	0,2	$MgSO_4.7H_2O$
	0,04	$MnSO_4.4H_2O$
	0,3	K_2HPO_4
	1,0	yeast extract
	0,5	proteose peptone

The pH was adjusted to 7,0, 12% gelatin added, 10 ml aliquots dispensed into test tubes and the medium sterilised. The medium was allowed to solidify in the slant position and inoculation was by a single streak.

Tryptone Yeast Extract Broth (xvii)

Shirling and Gottlieb (1966)

In g/l	5,0	Bacto-tryptone
	3,0	Bacto-yeast extract

The pH was adjusted to 7,2 and 100 ml aliquots dispensed into 250 ml conical flasks and sterilised.

Trypticase Soy Broth Agar (xviii)

In g/l	30	BBL trypticase soy broth
	15	Difco Bacto agar

The medium was autoclave sterilised and plates poured.

Yeast Extract Agar (xix)

Hendrie et al. (1968)

In g/l	1,0	yeast extract
	8,0	agar

The pH was adjusted to 7,0 and the medium sterilised.

Hugh and Leifson's Medium (xx)

Collins and Lyne (1984)

In g/l	2,0	peptone
	5,0	NaCl
	0,3	K_2HPO_4
	2,0	bromothymol blue
	10,0	dextrose
	3,0	agar

The agar was dissolved by boiling. pH was adjusted to 7,1. Medium dispensed into test tubes and sterilised.

McBeth's Cellulose Ammonium Sulphate (xxi)

Salle (1948)

In g/l	1	K_2HPO_4
	1	$MgSO_4.7H_2O$
	1	Na_2CO_3
	2	$(NH_4)_2SO_4$
	2	$CaCo_3$

The medium was dispensed, with continuous stirring, into test tubes containing strips of filter paper 10 mm x 10 mm, and sterilised.

Gram's Stain (xxii)

Staining reagents were made up according to Jenson's

version as described by Salle (1948).

Solution 1	0,5 g methyl violet dissolved in distilled water
Solution 2	2 g potassium iodide and 1 g iodine dissolved in less than 20 ml distilled water then made up to 100ml
Solution 3	1 g basic fuchsion dissolved in 100 ml 95% ethyl alcohol. The mixture was allowed to stand for 24 hours, then filtered and 900 ml distilled water added

<u>Cell Wall Fatty Acid Extraction Reagents</u> (xxiii)

Reagent 1	45	g	NaOH
	150	ml	methanol
	150	ml	deionised distilled water
Reagent 2	325	ml	6N HCl
	275	ml	methanol
Reagent 3	200	ml	hexane
	200	ml	methyl tri-butyl ether
Reagent 4	10,8	ml	NaOH
	900	ml	deionised distilled water

<u>Xylanase Assay Medium</u> (xxiv)

Ramsay and Fry (1976)

In g/l 20,0 casein hydrolysate

10,0	mycological peptone
15,0	soluble starch
3,0	xylan
10,0	bacteriological agar

The pH was adjusted to 7,2.

<u>Sodium polypectate gel</u> (xxv)

Hildebrand (1971)

To 1000 ml distilled water, heated to near boiling was added (in order):

1 ml bromothymol blue (1,5% alcoholic solution)

6 ml $CaCl_2.2H_2O$ (10% solution, freshly prepared)

22 g sodium polypectate

The medium was divided into 3 equal volumes and the pH's adjusted as follows:

Medium A: pH 4,9-5,1 using 1N HCl

Medium B: pH 6,9-7,1 using 1N NaOH

These media were then sterilised

Medium C: This medium was first sterilised and the pH then adjusted to 8,3-8,5 using sterile 1N NaOH

100 ml 4% agar, separately sterilised was added to each medium after autoclaving and plates were poured while the media were above 70°C.

<u>Pectate lyase Induction Medium</u> (xxvi)

Zucker and Hankin (1970)

In g/l		
	2	$(NH_4)_2SO_4$
	4	KH_2PO_4
	6	Na_2HPO_4
	0,001	$FeSO_4.7H_2O$

	0,2	$MgSO_4.7H_2O$
	0,001	$CaCl_2$
In mg/l	10	H_3BO_3
	10	$MnSO_4$
	70	$ZnSO_4$
	50	$CuSO_4$
	10	MoO_3

The pH was adjusted to 7,4 and 12,5 ml aliquots of the mineral medium dispensed into 250 ml conical flasks. 6,25 ml distilled water and approximately 1,7g freshly cut potato discs were added to each flask. The carbon source, polygalacturonic acid, was prepared separately as a 4% solution. After autoclaving of the mineral medium and of the carbon source, 6,25 ml of the carbon source was added aseptically to each flask thus achieving a final concentration of 1%.

Pectin Methylesterase Induction Medium (xxvii)

The mineral medium used for pectate lyase induction medium (Zucker and Hankin, 1970) was made up as previously described and the pH adjusted to 7,4. 12,5 ml aliquots of the medium were dispensed into 250 ml flasks and 6,25 ml distiled water added to each. The carbon source Rapid set citrus pectin (H.P. Bulmer, Hereford, England) was prepared separately as a 4% solution. The mineral medium was autoclaved at 121°C for 15 minutes and the carbon source at 121°C for 4 minutes. 6,25 ml of carbon source was then added

aseptically to each flask thus achieving a final concentration of 1%.

Cellulase Induction Medium (xxviii)

Reese and Mandels (1963)

In g/l	2,0	KH_2PO_4
	1,4	$(NH_4)_2SO_4$
	0,3	urea
	0,3	$MgSO_4.7H_2O$
	0,3	$CaCl_2$
	1,0	proteose peptone (Difco)
	10,0	solka floc (Whatman)
In µg/l	5,0	$FeSO_4$
	1,6	$MnSO_4.H_2O$
	1,7	ZnCl
	2,0	$CoCl_2$

The pH was adjusted to 6,9, 100 ml aliquots dispensed into 500 ml conical flasks and the medium sterilised.

6. SAMPLING FROM PACKS

Packs were sampled in a variety of different ways.

a) Culture

Direct Plating (i)

Organisms adhering to the leaf or other surfaces were removed by paddling in a stomacher (A J Seward Lab-Blender 400 model BA 6021) as described by Fry and Humphrey (1978). The leaves from one leaf pack were placed into a sterile stomacher bag with 50 ml sterile distilled water. In order to

protect the sample bag from bursting during paddling it was placed into another stomacher bag containing 200 ml water. This acted as a buffer. The sample was paddled for 5 minutes to produce a suspension of microorganisms.

The suspension was serially diluted in sterile water and plated directly onto Oxoid Nutrient agar (NA) and incubated at 15°C for up to 6 weeks.

Enrichment Techniques (ii)

1 ml aliquots of undiluted suspension were inoculated into liquid enrichment media and incubated on a Gallenkamp orbital shaker at 150 rpm and 15°C for 2 weeks. Individual leaves from a pack were added to each of several 100 ml aliquots of distilled water in 500 ml flasks. The flasks also contained either one autoclave-sterilised alder leaf (approximately 0,2 g) or one strip of sterile cellophane 20 mm x 60 mm. The flasks were incubated on an orbital shaker as described above.

Other leaves from the same pack were placed into each of several test tubes (28 mm x 170 mm) containing 20 ml distilled water and a cellophane wick as described by Willoughby (personal communication) (see fig. 1) and incubated at 15°C for 2 weeks.

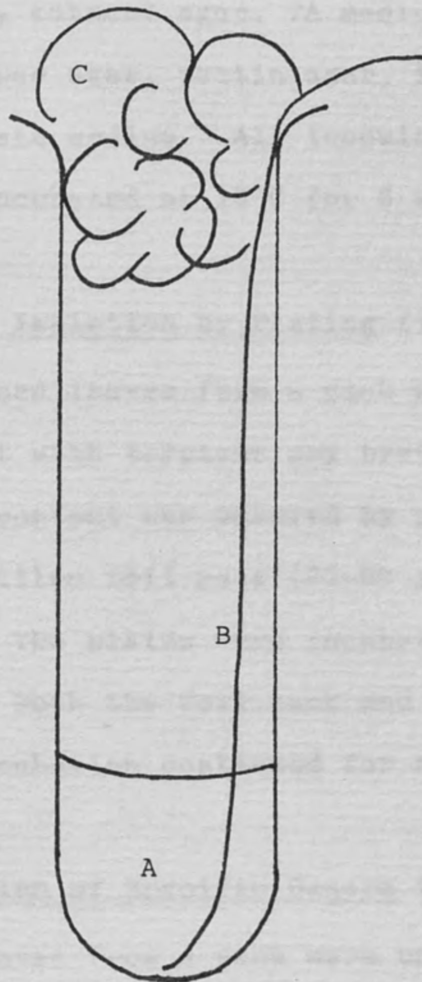


Figure 1: Construction of a cellophane wick apparatus for selective isolation of microorganisms. A 28mm x 170mm test tube contains 20ml sterile distilled water (A) and a sterile cellophane wick (B) and is sealed with cotton wool (C).

Where organisms were first removed from the leaf or cellophane surfaces by stomaching, enrichment suspensions were diluted and plated out onto the following selective media: peptone succinic acid medium, oatmeal agar, TA medium, chitin agar, cellulose agar, pectin agar, leaf homogenate agar and paste medium. All inoculated selective media were incubated at 15°C for 6 weeks.

Direct Isolation by Plating (iii)

Colonised leaves from a pack were placed in direct contact with tryptone soy broth agar (TSBA). A close contact was ensured by placing a sterile sand-filled foil pack (60-80 g) on top of the leaf. The plates were incubated for 24 hours at 15°C. Both the foil pack and leaf were removed and incubation continued for a further 6 weeks.

Isolation of Specific Genera (iv)

The leaves from a pack were used to isolate specific genera such as *Cytophaga*, *Streptomyces* and *Actinoplanes* using the method described by Makkar and Cross (1982). To reduce numbers of non-spore forming microorganisms the leaves were air dried at room temperature on paper towels. After 10 days each leaf was rehydrated separately in 50 ml tap water in 100 ml conical flasks. The flasks were shaken periodically during the one

hour rehydration process. Suspension was then plated out onto chitin agar and tap water agar and incubated at 15°C for up to 6 weeks with regular inspection.

Growth of Actinoplane spp was encouraged by using dog fur as a substrate rich in keratin, which some members of this genus are known to degrade (Makkar and Cross, 1982). A colonised leaf, dried as before, was placed into 50 ml sterile tap water in 250 ml conical flasks, to which was added a small quantity (less than 0,1 g) sterile dog fur. The flasks were incubated on a Gallenkamp orbital shaker at 220 rpm and 15°C. After 6 weeks the culture was plated onto chitin agar. Dog fur from the culture flask was sampled by direct placing of a few strands onto both chitin agar and damp filter paper in a petri dish as described by Willoughby (1968). The latter was sealed with tape and the plates were incubated at 15°C for 6 weeks. Strands of dog fur were viewed under phase contrast on a Leitz Diaplan microscope to ascertain whether or not Actinoplane spp were present.

All isolates were purified by streaking onto the appropriate solid media. Pure cultures were maintained on NA, TSBA or yeast-extract malt-extract agar slants at 4°C.

7. DIRECT VIEWING OF COLONISED ORGANISMS

Scanning electron microscopy and fluorescence microscopy were used to view the organisms on the surfaces of the leaves which had been incubated in packs, in the river.

8 discs were randomly cut from the leaves from the pack using a number 5 corkborer. 3 discs were processed for scanning electron microscopy and the remaining 5 for fluorescence microscopy.

Scanning Electron Microscopy (i)

Scanning electron microscopy was used to view bacterial morphology.

Method

Fixation

Samples were fixed using one of the following methods:

- 8 hours in 2,5% glutaraldehyde.
- 4 hours in 2,5% osmium tetroxide.
- 20 minute exposure to osmium tetroxide vapour (King and Brown, 1983).

Leaf discs were placed onto the interior surface of a glass petri dish lid (48 mm x 15 mm). This lid was inverted over the petri dish bottom (45 mm x 15 mm) containing 5 ml aqueous 1% osmium tetroxide previoulsy warmed to room temperature. Following a 20 minute exposure to the osmium tetroxide vapour, the lid was removed and placed over a second petri dish bottom containing 5-8 ml distilled water warmed to 35-45°C.

Dehydration

Samples were dehydrated usisng one of the following methods:

Samples were washed in 3 changes of phosphate buffer pH 7,2 for 10 minutes each and dehydrated in a graded acetone series as detailed below:

50% acetone 2 changes of 5 minutes each

70% acetone 2 changes of 5 minutes each

95% acetone 2 changes of 5 minutes each

100% acetone 3 changes of 10 minutes each

or

Samples were gradually dehydrated under slight vacuum through a vapour diffusion dehydration (VDD) process as described by King and Brown (1983). The VDD apparatus consisted of a glass vacuum dessicator containing acetone over aluminium silicate molecular sieves 10-16 mesh (Aldrich) as a dessicant (see fig. 2). The dessicator contained a perforated aluminium support plate, and a large crystallizing dish, which was inverted on the support plate, and on top of which was placed a glass petri dish containing fresh indicator silica gel.

The fixed specimens, if not already in a petri dish, were placed into glass petri dish lids. A few drops of distilled water were added to each lid which were then placed in the dessicator on the support plate below the

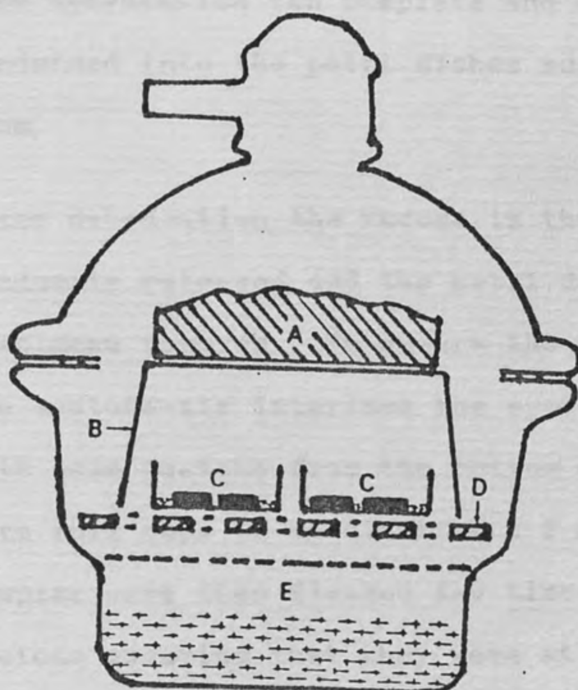


Figure 2: Construction of a vacuum dessicator assembly for Vapour Diffusion Dehydration (VDD) of SEM samples. A 10cm glass petri dish bottom (A) contains dry indicator silica gel; a flat-bottomed dish (B) is inverted over 4cm glass petri dish covers containing the fixed specimens and distilled water (stippled); D, a perforated metal plate; E, absolute acetone over alumina molecular sieves to a level approximately 1cm below the support plate. From King and Brown (1983). Can J. Microbiol., 29.

inverted crystallizing dish. The dessicator lid was positioned and a vacuum sufficient to allow the acetone to just boil was drawn. The exhaust port was then closed and the dessicator left overnight. After this time dehydration was complete and acetone vapour had condensed into the petri dishes sufficient to fill them.

After dehydration the vacuum in the dessicator was gradually released and the petri dishes containing the specimens removed. To ensure the minimum contact with the acetone-air interface the specimens were scooped with some acetone from the bottom of the petri dishes into foil cups (5 mm diameter x 5 mm high). The samples were then flushed 4-5 times with absolute acetone ensuring that they were at all times submerged.

Critical Point Drying

Samples were critical point dried for 3 hours under carbon dioxide in an E3000 Polaron Critical Point Dryer and mounted on 13 mm aluminium stubs (Agar Aids) using double sided tape.

Coating

Samples were coated with 2 x 20 nm thick layers of gold palladium in a Polaron E5100 Series II 'Cool' Sputter Coater. Samples were stored in a dessicator at room temperature.

Scanning Electron Microscope Viewing

Samples were viewed either under a Jeol JSM-255 or a Cambridge S100 scanning electron microsocpe.

Fluorescence Microscopy (ii)

Fluorescence microscopy was used to count organisms on the surface of leaves. Leaf discs were stained in 10 mg/l acridine orange for 3-5 minutes (Fry and Humphrey, 1978) and examined with a Leitz x 50 or x 100 oil immersion objective in a Leitz Diaplan microscope. The stained samples were illuminated with a UV lamp with a Leitz Hz filter block. Bacteria fluoresced green or orange. Five randomly selected fields were counted on each of the 5 sample discs and the results expressed as number of bacteria/cm^2 of the sample.

Direct enumeration by fluorescence microscopy of river water organisms colonising sterile alder leaf discs *in vitro* (iii)

Bacteria colonising sterile alder leaf discs incubated in a closed system continuous flow fermentor were enumerated by fluorescence microscopy.

The apparatus consisted of a 1000 ml bioflo fermenter fitted with a side arm containing a downwards facing outlet port and an L-shaped vent which protruded above the water level in the fermentor and beyond the outlet port within the side arm and functioned to drain excessive water buildup in the event of airlocking (fig. 3). The fermentor contained an internal metal

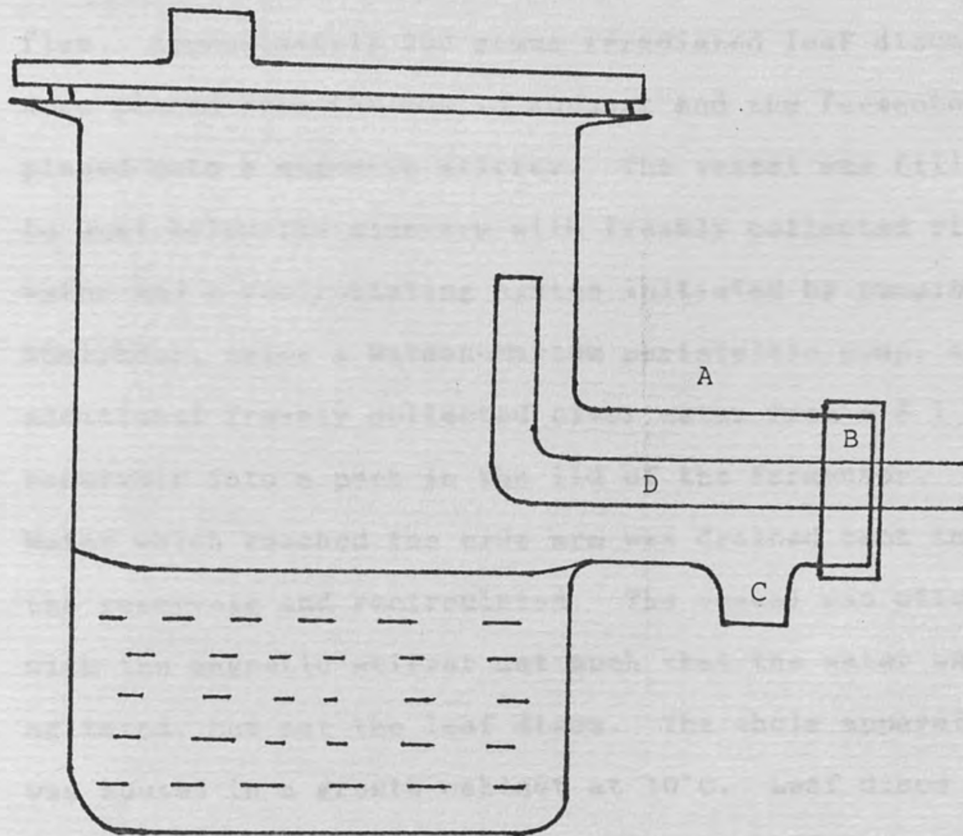


Figure 3: Construction of a sidearm with vent in a fermentor to drain excess water in the event of airlocking. A fermentor vessel is fitted with a sidearm 2cm in diameter (A) which has a screw thread at the distal end (B). An L-shaped glass tube, 1cm in diameter (D) is placed such that its one end faces upwards inside the fermentor vessel and the other passes through the sidearm and is secured and sealed with a plastic cap with centrally placed hole which fits over the smaller glass tube.

frame onto which was fixed a sheet of muslin which allowed the leaf discs to be suspended above a magnetic flea. Approximately 300 gamma irradiated leaf discs were placed onto the muslin support and the fermentor placed onto a magnetic stirrer. The vessel was filled to just below the side-arm with freshly collected river water and a recirculating system initiated by pumping 50ml/hour, using a Watson-Marlow peristaltic pump, of additional freshly collected river water from a 5 l reservoir into a port in the lid of the fermentor. Water which reached the side arm was drained back into the reservoir and recirculated. The vessel was stirred with the magnetic stirrer set such that the water was agitated, but not the leaf discs. The whole apparatus was housed in a growth cabinet at 10°C. Leaf discs were removed with sterile forceps at regular intervals for enumeration of colonised organisms by staining with acridine orange and viewing under ultraviolet light.

8. IDENTIFICATION OF ISOLATES

a) General Testing

Colony Pigmentation (i)
Growth was inspected visually under daylight conditions, and under ultra violet in a darkened area for production of fluorescent pigments.

Cell Morphology and Arrangement and Motility (ii)
Loopfuls of 24 hour old agar plate cultures were

suspended in a drop of distilled water on a microscope slide and viewed under phase contrast on a Leitz Diaplan Microscope. Additionally hanging drop suspensons of 24 hour old nutrient broth (NB) cultures were viewed. Presence or absence of motility was recorded.

Gram Stain (iii)

Jensen's version of the Gram stain was used

The heat fixed samples were stained in solution 1 for 30 seconds. Solution 1 was rinsed off and solution 2 applied and left for 1 minute. The preparation was decolourised with 95% alcohol and counterstained with solution 3 for 10-30 seconds. The slides were drained and blotted dry.

Carbohydrate Fermentations (iv)

Media were prepared using commercially produced Purple Broth Base (Oxoid) with 1% appropriate carbohydrate added. Aliquots of approximately 10 ml were dispensed into test tubes either fitted with Durham tubes (glucose fermentations) or without and sterilised by autoclaving. Tubes were inoculated with loopfuls of culture from solid media and incubated at 25°C for 3 days with daily inspection.

Anaerobic Growth (v)

Isolates were inoculated onto NA or TSBA plates

and incubated under anaerobic conditions in a BBL anaerobic jar for 7 days at 25°C.

Catalase Production (vi)

A few drops of 3% hydrogen peroxide were applied to growth on an agar plate and observed for effervescence.

Oxidase Production (vii)

Loopfuls of culture from solid media were smeared onto filter paper onto which a drop of freshly prepared 1% tetramethyl-p-phenylene diamine dihydrochlorine was placed. The sample was observed for formation of a purple colour.

Indole Production (viii)

Loopfuls of culture from solid media were inoculated into Tryptone Broth and incubated for up to 4 days at 25°C or for a shorter period of time if sufficient growth was obtained. 5 ml of Kovac's reagent was added to each tube which were then observed for formation of a deep cherry red colour.

Citrate Utilization (ix)

Slants of Simmon's citrate agar were inoculated with growth from solid agar and incubated at 25°C for 72 hours with daily inspection.

Starch Hydrolysis (x)

Plates of starch agar were inoculated by streaking

of cultures and incubated at 25°C for 48 hours after which they were flooded with a dilute iodine solution in order to visualise areas of starch breakdown.

Gelatin Liquefaction (xi)

Loopfuls of 24 hour NB culture were inoculated onto gelatin medium and incubated at 25°C. Tubes were regularly inspected over 2 weeks incubation period for gelatin liquefaction.

Oxidative-fermentative Metabolism (xii)

Isolates were inoculated in duplicate into tubes of Hugh and Leifson's O/F medium, one of which was overlaid with mineral oil. Samples were incubated at 25°C for 3 days with daily inspection.

Flagellar Number and Insertion (xiii)

Organisms were cultured for 24 hours in tubes of NB, centrifuged at 1000 g for 5 minutes, washed twice and resuspended in less than 1 ml sterile distilled water. A drop of this suspension was placed onto a 3 mm, 300 mesh formvar coated grid, allowed to dry and overlaid with 2% potassium phosphotungstate with 4% added sucrose. Excess potassium phosphotungstate was removed using strips of filter paper. Grids were viewed in a Zeiss 109 transmission electron microscope and organisms photographed onto Ilford HP4 print film.

Digestion of Filter Paper Strips (xiv)

Salle (1948)

Isolates were inoculated in duplicate into tubes of McBeth's cellulose and ammonium sulphate solution. One tube was overlaid with vaspar and both tubes incubated at 25°C for 6 weeks with regular inspection.

b) Testing of Putative Xanthomonas spp

The methods used were described by Hendrie et al. (1968).

Spreading Growth Pattern (i)

Isolates were inoculated onto yeast extract agar plates, incubated at 25°C and resultant growth inspected for spreading behaviour.

Carbohydrate Fermentation (ii)

Isolates were inoculated onto slopes of Dye's medium C and incubated at 25°C for 3 days with daily inspection.

Bacteriolytic Activity (iii)

Escherichia coli grown on NA slant was suspended in distilled water and washed twice. This suspension was spread onto the surface of water agar plates which were then dried at 45°C for 4-5 hours. Isolates were then streaked onto the medium and the plates were incubated at 25°C for 4 weeks and regularly inspected for bacteriolytic activity.

Polymyxin B Sensitivity Testing (iv)

Polymyxin B sensitivity testing discs were made up according to the method described by Collins (1964). Discs of filter paper 5-6 mm in diameter were cut with a cork borer, spread over the surface of a glass petri dish and sterilised in a hot oven. A solution of polymyxin B was prepared in distilled water such that there were 100 units per 0,02 ml which is the volume absorbed by a 5-6 mm filter peper disc. Discs were dipped into the antibiotic solution and dried in a glass petri dish in a vacuum dessicator over phosphorous pentoxide.

c) Cell Wall Fatty Acid Extraction and Profiling

Extraction

Isolates were quadrat-streaked onto trypticase soy broth agar and incubated at 28°C. After 24 hours ± 1 hour cells were harvested, using a flamed aluminium spatula, into clean glass screw top test tubes fitted with teflon-lined caps. Cell wall fatty acids were extracted as follows:

Saponification

1 ml of reagent 1 was added, tubes vortexed and placed in a 100°C water bath. After 5 minutes tubes were again vortexed and incubated for a further 25 minutes, then removed and cooled at room temperature.

Methylation

2 ml of reagent 2 was added to each sample which was then vortexed and placed in a water bath at 80°C. After 10 minutes tubes were removed and cooled rapidly on crushed ice.

Extraction

1,25 ml reagent 3 was added to each sample. Samples were rotated end over end for 10 minutes in an end over end shaker (Gerhardt, supplied by Camlab, Cambridge). The bottom phases were removed, using a pasteur pipette, and discarded.

Base-wash

3 ml of reagent 4 was added to the tubes which were then rotated end over end for 5 minutes.

The top phases were removed, using pasteur pipettes, and placed into gas chromatographic sampler vials which were sealed with aluminium/rubber cups using a cap crimper. If the top phase appeared cloudy a few drops of saturated NaCl were added to clear it, prior to its removal.

Gas Chromatography

Gas chromatographic parameters for separation of fatty acid methyl eshers were as follows:

Carrier gas	hydrogen
Auxilliary gases	nitrogen

Initial temperature	170°C
Final temperature	270°C
Programme rate	5°C/minute
Injection port temperature	250°C
FID temperature	300°

Fatty acid methyl esters were separated by a Hewlett Packard 5890A Gas Chromatograph fitted with a 25m fused silica capillary column coated with methyl phenyl silicone. Peaks were integrated on a Hewlett Packard 3392A integrator and further identified both individually and as profiles by comparison with a library of bacterial cell wall fatty acid profiles by the Hewlett Packard 5898A Microbial Identification System software package 19298A. The system was standardised by injection of a standard calibration mix which contained 9C-20C straight chain saturated esters and several -2 and -3 hydroxy esters (Microbial I.D. Inc., Suite 115, Barksdale Professional Center, Barksdale Road, Newark, Delaware, 19711, USA).

d) API 20NE Bacterial Identification Strips

Gram negative oxidase positive organisms were inoculated onto API 20NE (API system S.A. Basingstoke, UK) strips according to manufacturers instructions. Results were read after 24-48 hours incubation at 30°C and resulting numerical profiles identified by comparison with the index

of bacterial profiles as supplied by the manufacturers.

9. SUCCESSION OF BACTERIA ON DECAYING ALDER LEAVES - IN VITRO STUDY

An attempt was made to investigate the succession of representatives of 9 different bacterial genera, isolated in the field study, on decaying alder leaves *in vitro*. Known concentrations of these genera were inoculated into basal medium with sterile leaves and the pattern of colonisation monitored at selected time intervals by subculture of the colonising organisms on selective media. Colonising organisms were also viewed directly using scanning electron microscopy.

To each of 100 ml basal medium in 500 ml conical flasks was added 1-3 sterile dried alder leaves and 5-10 sterile 5 mm dried alder leaf discs. This medium was inoculated with a mixture consisting of 1 ml each of overnight NB cultures of each of the organisms used. A viable count at the start of the experiment was made by plating out samples of the mixture onto selective media (see table 7).

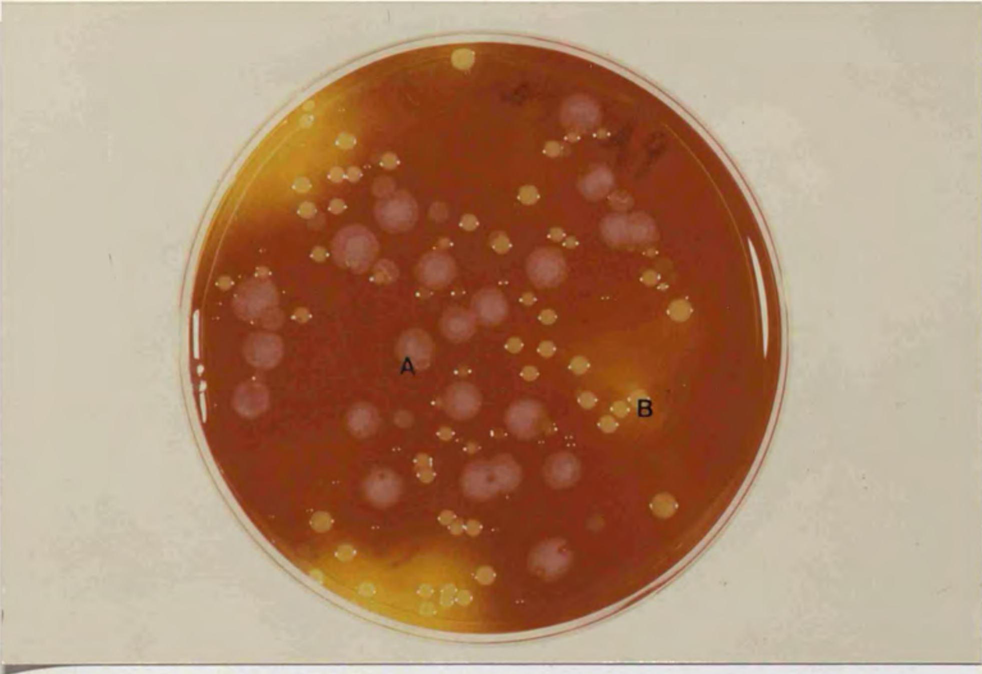
The selective media allowed simple, unambiguous identification of the constituent genera on the basis of pigment production or on colony morphology (see figures 4-8).

Genus	Selective medium	Length of incubation time	Mode of enumeration
Bacillus	YEME	48h	direct count
Pseudomonas	PAA	48h	direct count
Aeromonas	PAA	48h	direct count
Klebsiella	MacConkey agar	24h	direct count
Alcaligenes	MacConkey agar	24h	direct count
Rhodococcus	NA	96h	direct count
Xanthomanas	NA	48h	direct count
Streptomyces	CA	7-10 days	direct count
Erwinia	NA	4h	By difference - deducting total numbers of all genera except streptomyces from total count on nutrient agar

Table 7: Selective media, length of incubation time and method by which organisms removed from decaying alder leaves *in vitro* were enumerated.

Figure 4: Typical growth obtained on PAA from spread plate inoculation with a defined mixed culture. *Pseudomonas* colonies (A) are pink and *Aeromonas* colonies (B) yellow.

Figure 5: Typical growth obtained on CA from spread plate inoculation with a defined mixed culture. *Streptomyces* colonies (A) have aerial mycelium and grow in concentric rings.


A
B

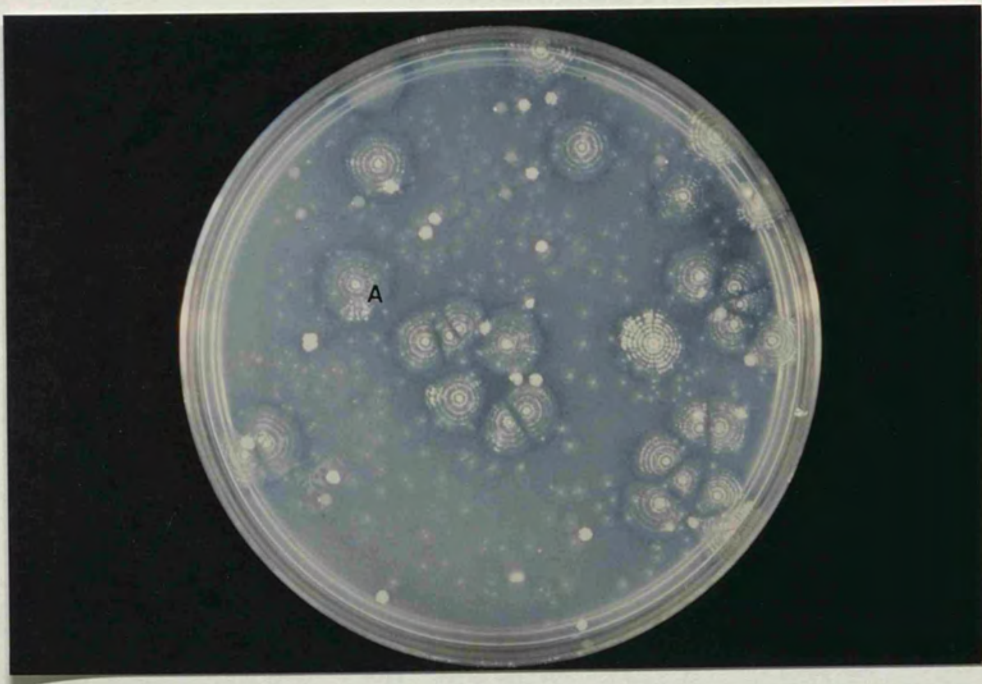

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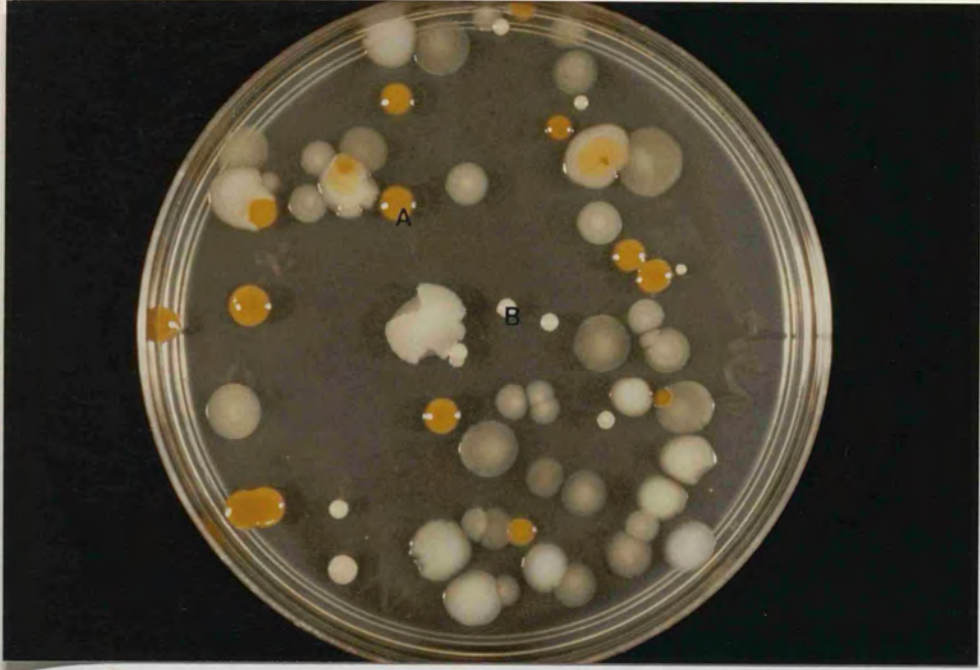
Figure 6: Typical growth obtained on NA from spread plate inoculation with a defined mixed culture. _Xanthomonas_ colonies (A) are yellow and _Rhodococcus_ colonies (B) coral pink and raised.

Figure 7: Typical growth obtained on MacConkey agar from spread plate inoculation with a defined mixed culture. _Klebsiella_ colonies (A) are pink and mucoid and _Aeromonas_ colonies (B) are buff-coloured and small.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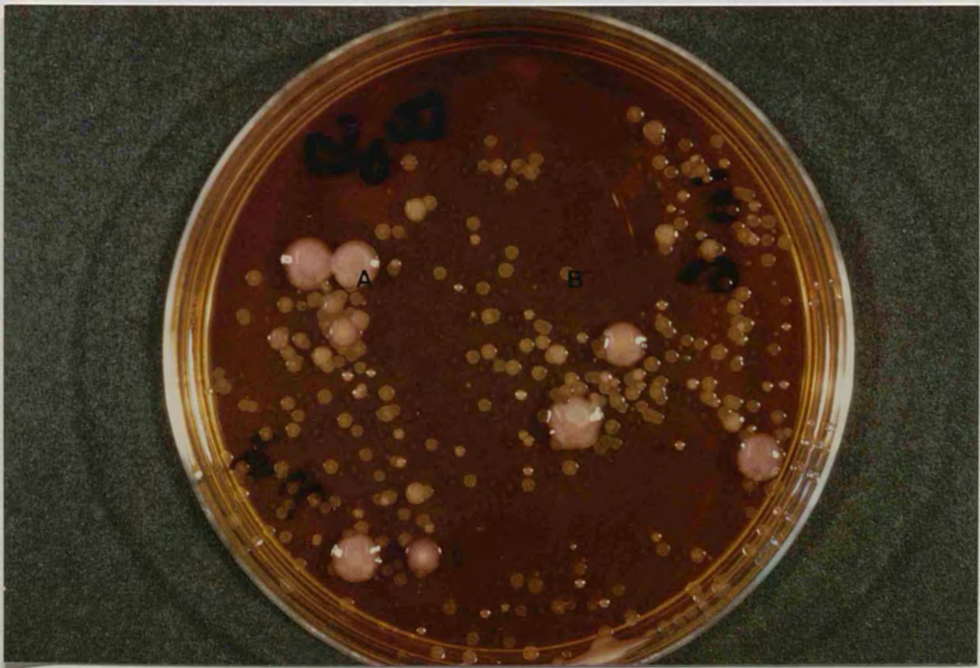
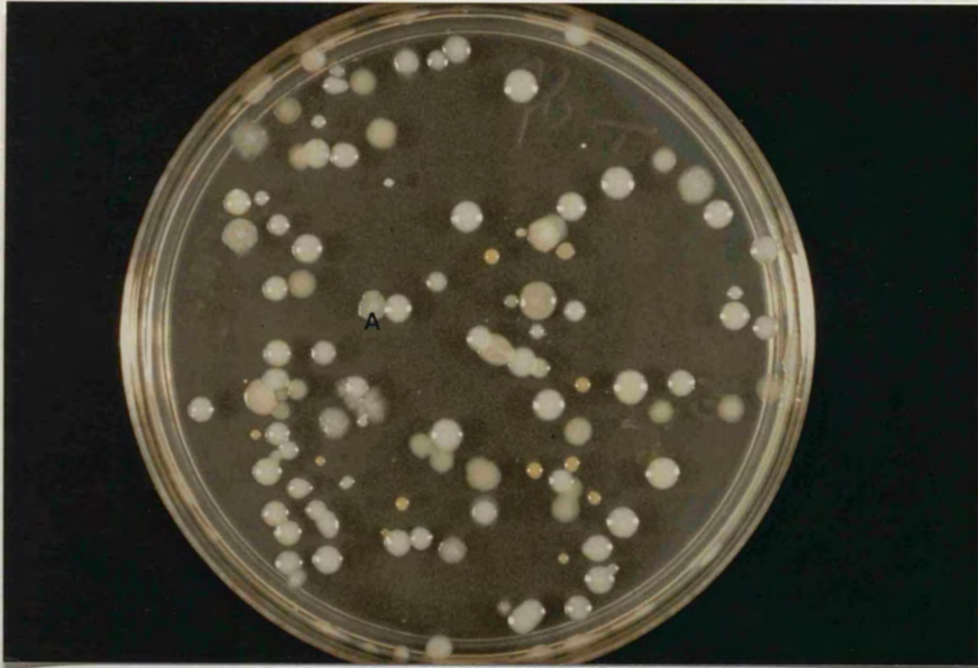

A
B

Figure 8: Typical growth obtained on YEME from spread plate inoculation with a defined mixed culture. _Bacillus_ colonies (A) are off-white coloured, flat and are striated.

Cultures were incubated on a Gallenkamp orbital shaker at 220 rpm and sampled daily for the first 3 days and thereafter weekly. Two cultures designated A and B, were always sampled.

The colonised leaves from one culture were placed into 500 ml sterile distilled water in a 1000 ml beaker and gently swirled in order to remove non-adherent organisms. Adherent organisms were removed by paddling in a stomacher as described in section [illegible]. A count



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The colonized leaves from the second culture were treated in the same manner used for the first. The same beaker of distilled water used to remove non-adherent organisms from leaves in culture A, was used for culture B.

The leaf discs also present in culture flasks were removed from one of each pair of cultures sampled and processed for scanning electron microscopy using 2.5%

Cultures were incubated on a Gallenkamp orbital shaker at 220 rpm and sampled daily for the first 3 days and thereafter weekly. Two cultures designated A and B, were always sampled.

The colonised leaves from one culture were placed into 500 ml sterile distilled water in a 1000 ml beaker and gently swirled in order to remove non-adherent organisms. Adherent organisms were removed by paddling in a stomacher as described in section 6a(i). A count of organisms in the suspension was carried out using a Thoma Helber counting chamber and served to indicate which dilutions, when plated, would yield countable numbers of colonies. The suspension was arbitrarily designated the 10^{-1} dilution. in each case 5 consecutive dilutions were plated in 0,1 ml aliquots onto the media using the spread plate method. Plates were incubated at 25°C overnight and then on the bench in daylight at room temperature in order to allow pigments to develop.

The colonised leaves from the second culture were treated in the same manner used for the first. The same beaker of distilled water used to remove non-adherent organisms from leaves in culture A, was used for culture B.

The leaf discs also present in culture flasks were removed from one of each pair of cultures sampled and processed for scanning electron microscopy using 2,5%

glutaraldehyde to fix samples and a graded series of acetone for dehydration. Samples were viewed under a Cambridge S100 scanning electron microscope.

10. LEAF POLYMER CONTENT

The total percentage pectin, cellulose and hemicellulose content of dried alder leaves was determined using the method of Jermyn and Isherwood (1956). The cell walls contained in the dried alder leaves were extracted prior to measurement of polymer content.

Extraction of Cell Walls

after Laborda et. al. (1973) with modifications. The leaves were crushed by hand in a polythene bag and dropped into acetone at -20°C. One volume of leaves and 3 volumes of acetone were blended in a Waring blender at full speed for 3 minutes. The subsequent slurry was filtered and the filtrate was treated with a further 3 volumes of acetone or until the filtrate was free of pigment. The filtrate was then homogenised in 10 times its volume 0,1 M potassium phosphate buffer at pH 7,0 for 5 minutes. The solid matter was filtered off through a course glass sinter and resuspended in a further quantity of buffer. This process was repeated a further 4 times with buffer and 5 times with distilled water, at 4°C. The filtrates were clear once this step was complete. The remaining solid matter, which was largely cell wall, was blended in 10x its volume chloroform: methanol (1:1 v/v) and filtered

through Whatman no 41 paper. The filtrate was washed with chloroform: methanol (1:1) and finally in analytical grade acetone. The preparation was then dried at 60°C, cooled in a dessicator, ground up using a pestle and mortar and stored at room temperature.

Pectin

10g of the cell wall preparation was extracted in 750 ml water maintained at 98-100°C in a heating mantle for 12 hours. The extract was filtered through sintered glass and washed with 6 x 200 ml boiling water. The residue was mixed thoroughly with acetone and washed on the filter with more acetone. The residue was dried in an air current, weighed and the percentage pectin calculated as the weight lost from the 10 g sample.

Delignification

5 g of the above dried, pectin free residue was suspended in 160 ml water in a 250 ml conical flask to which was added 10 drops of acetic acid and 1,5 g sodium chlorite. The flask was sealed with a lightly fitting ping pong ball and immersed in a 75°C water bath to a point above the level of the liquid in the flask. At hourly intervals 10 drops of acetic acid and 1,5 g sodium chlorite were added. The sample was treated for 4 hours and then filtered through sintered glass. The flask was washed with several portions of iced water in order to remove adhering matter and the contents of the sintered glass funnel were washed 10 times with iced water followed by acetone. The residue

was dried in an air current and the percentage of lignin calculated as the weight lost from the 5 g sample. The fraction remaining was the holocellulose.

Hemicellulose A Extraction

2,5 g holocellulose was placed into a 125 ml conical flask with 100 ml N-KOH. The flask was sealed with a rubber bung and maintained at 20°C for 2 hours with gentle shaking every 10 minutes. Contents were filtered through sintered glass, washed and the residue dried in an air current. Hemicellulose A was calculated as the percentage weight lost from the 2,5 g sample.

Hemicellulose B Extraction

The residue of the hemicellulose A extraction was placed into a 125 ml conical flask with 4N KOH. The flask was sealed with a rubber bung and maintained at 20°C for 2 hours with gentle shaking every 10 minutes. The contents were filtered through sintered glass and the residue washed with 25 ml 4N KOH. 250 ml water and 25 ml 2N acetic acid and the residue dried in an air current. Hemicellulose B content was calculated as the percentage weight lost from the sample.

Cellulose Content Calculation

The percentage cellulose present in dried alder leaves was calculated as the residue remaining after extraction of hemicellulose A and B.

11. SUBSTRATE UTILIZATION

The ability of isolates to liquefy pectate gel at 3 different pHs was tested. Organisms from overnight NB cultures were point inoculated onto the surface of sodium polypectate gel at pH 4,95, 7,04 and 8,36 and incubated at 25°C for 24 hours or until visible growth had occurred. Plates were developed with 1% hexadecyltrimethylammoniumbromide (cetrimide) in order to view areas of substrate breakdown. Breakdown of remazol brilliant blue-dyed cellophane strips was used to demonstrate visually cellulase production. Cellophane was dyed according to the method as described by Moore et al. (1979) as follows:

100 20 mm x 50 mm non-moisture proof cellophane strips were boiled in 2 changes of 500 ml distilled water and then slowly heated, while kept in suspension, in a further 500 ml distilled water. At 80°C, 1,5 g Remozol brilliant blue (Aldrich) was added followed by 5 x 20 ml aliquots of 3% aqueous Na_2SO_4 added at 2 minute intervals and finally 2,50 g $Na_3HPO_4.12H_2O$ in 15 ml water was added and the temperature maintained for 20 minutes. Strips were rinsed in water at 60°C until the washings were colourless and then autoclaved in 2 changes of 1000 ml distilled water each for 15 minutes. Strips were then air dried and stored in a sealed container.

One strip each of dyed cellophane was placed in 50 ml of the cellulose induction medium minus solka floc in

100 ml conical flasks and autoclaved. A replicate set of flasks containing 1% added cellobiose as an additional inducer was also prepared. These media were each inoculated with 5 ml of washed cells from overnight NB cultures and incubated on a Gallenkamp orbital shaker at 220 rpm and 25°C for 6 weeks. Release of the dye was considered to represent cellulase production.

Breakdown of xylan, as a representative of the hemicellulose fraction, was demonstrated by streak-inoculation of isolates onto xylanase assay medium. Plates were incubated at 25°C for 2 weeks and clearings in the medium surrounding growth was considered to represent xylanase activity.

(a) Enzyme Induction

Pectate lyase (i)
after Zucker and Hankin (1970)
250 ml conical flasks each containing 25 ml basal medium with sodium polypectate were inoculated with 5 ml washed cells of the appropriate culture to give approximately 2 x 10^8 cells/ml. Cultures were incubated at 30°C and 220 rpm on a Gallenkanmp orbital shaker for 16 hours. Supernatants were clarified by centrifugation at 6000 rpm for 10 minutes, and stored at -10°C.

Pectin Methylesterase (ii)

250 ml conical flasks each containing 25 ml basal medium (Zucker and Hankin, 1979) with citrus pectin were inoculated with 5 ml washed cells of the appropriate culture. Cultures were incubated, clarified and stored as for pectate lyase induction.

Cellulase (iii)

Cellulases were induced in a medium used by Reese and Mandels (1963) for induction of these enzymes in *Trichoderma viride*.

Washed cells from 5 ml overnight NB or tryptone yeast extract broth were added to 100 ml of the cellulase induction medium with solka floc. Cultures were incubated on a Gallenkamp orbital shaker at 220 rpm and 25°C. In order to ascertain approximately when cellulases were being produced, replicate cultures in cellulase induction medium containing RBB dyed cellophane were simultaneously incubated. Supernatants from solka floc inductions were tested for cellulase activity once release of RBB from cellophane in smaller flasks became evident. These supernatants were filtered through muslin, clarified by centrifugation at 4000 rpm for 10 minutes and stored as described for pectinases.

(b) Assay Methods

Pectate Lyase (i)

after Zucker and Hankin (1970)

0,1 ml enzyme containing supernatant was added to the following reaction mixture, both previously equilibrated at 30°C.

In 2,9 ml	100 µm	tricine buffer ph 8,8
	20 µm	$CaCl_2$ (freshly prepared)
	0,3%	sodium polypectate

Change in absorbance at 235 nm and was measured in an LKB ultrospec 4050 spectrophotometer or a Philips PU8700 series UV/visible spectrophotometer. An increase in absorbance of 1,73 represented formation of 1 µmole of unsaturated uronide. One unit of enzyme activity was taken to be the amount that catalyzed the formation of unsaturated uronide at a rate of 1 µmole/minute at 30°C.

The ability of supernatants from the pectate lyase inductions to effect breakdown of sodium polypectate gel (Hildebrand, 1971) at different pHs was tested by inoculation of small aliquots into precut wells in the media. After 24 hours incubation at 25°C plates were developed with 1%

cetrimide in order to visualise areas of pectin breakdown.

Pectin methylesterase (ii)

after Delincée and Radola (1970)

To 20 ml of an unbuffered solution of 1% citrus pectin, pH adjusted to 9,0, was added 6 ml enzyme supernatant. The mixture was incubated in a water bath at 30°C and the change in pH measured every 10 minutes for 30 minutes. The supernatant from uninoculated induction medium was incubated under the same conditions and the change in pH due to enzyme activity took into account the changes in the substrate due to spontaneous hydrolysis of the pectin. Enzyme activity is expressed as $\triangle$pH/minute.

Cellulase (iii)

after Reese and Mandels (1963)

1 ml of enzyme-containing supernatant was added to 1 ml 0,55% $_{60}$T carboxymethylcellulose in 0,055M citrate buffer ph5,0 and incubated at 50°C for 90 minutes and reducing sugar concentration determined as follows:

Reagent	1%	3,5 Dinitrosalicylic acid
	0,2%	phenol
	0,05%	sodium sulphite
	1%	NaOH
	20%	sodium potassium tartrate

3 ml of the reagent was added to 1 ml of the enzyme/substrate mixture and the entire mixture was then boiled for 15 minutes in order to develop the colours.

Optical density was read at 550 nm in a Griffin Student Colorimeter using a buffer blank and concentration of reducing sugars calculated on a standard curve constructed using 0-0,4 mg glucose/ml. One enzyme unit was taken to be the amount which produced 4 mg reducing sugar in 10 ml of the reaction mixture.

(c) Dry weight Reduction

The reduction in dry weight of alder leaves caused by the action of microorganisms was tested by inoculation of 10 ml of washed cells into 100 ml aliquots of cellulase induction medium minus solka floc in 500 ml conical flasks containing 1 g of sterile preweighed alder leaves. Flasks were incubated on a Gallenkamp orbital shaker at 25°C and 220 rpm.

Uninoculated controls containing 0,2% sodium azide were simultaneously incubated. After 25 days the contents of each flask was filtered through 2 layers of dental muslin and dried to constant weight at 40°C.

The significance of the loss in dry weight of the

leaves incubated with microorganisms compared with sterile controls was analysed by one way analysis of variance and Turkey's Honest significant Difference testing (HSD) (Snedecor, 1956).

RESULTS

1. SEM study of succession of morphotypes on decaying alder leaves in freshwater

There were nine distinctly different morphotypes regularly observed on decaying alder leaves. These were:

1. Yeast cells (fig. 9)

   Ovoid or elongated cells approx. 10 µm x 7 µm and 14 µm x 5 µm respectively. Frequent budding observed.

2. Cocco-bacilli (fig. 10)

   Ovoid cells approx. 1-2 µm x 0,5-1 µm. Observed in isolation, in pairs, in groups or pairs within groups.

3. Intermediate length bacilli (fig. 11)

   Rod-shaped cells approx. 4 µm x 1 µm observed in isolation, in groups and in chains.

4. Flexuous bacilli (fig. 12)

   Any unbranched organism longer than 4 µm which displayed a wavy or kinked morphology.

5. Coccoid (fig. 13)

   Any spherical organism with a diameter less than 2 µm. Observed in pairs or small groups.

6. Prosthecate (fig. 14)

   Rod-shaped organisms with stalks. Sometimes observed dividing

7. S-shaped organisms (fig. 15)

   Organisms appearing as a single letter S, approx. 8 μm long.

8. Chains or pairs of flexuous bacilli separated by what appear as discs (fig. 16).

9. Brick-shaped bacilli (fig. 17)

   Chains of rod-shaped cells with squared corners. Individual cells are approx. 3 μm x 1 μm.

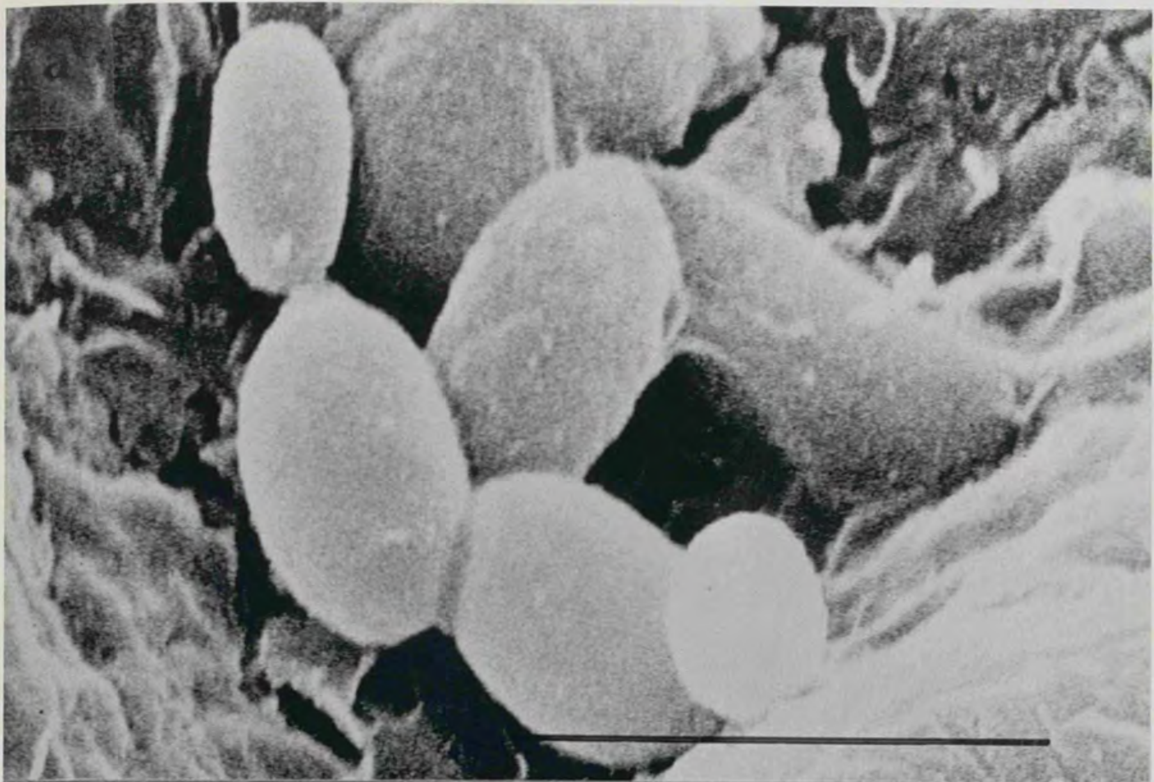
Samples were all scanned for 2½ to 3 hours and representative morphotypes photographed. Abundance of morphotypes was rated on a scale of 1 to 4 plus signs (tables 8-12).

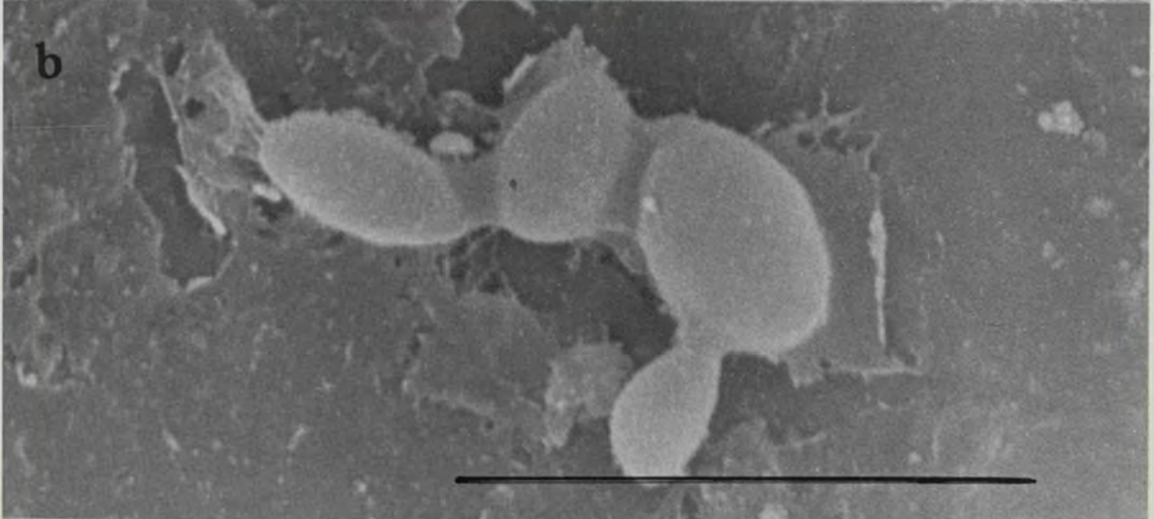
Yeast cells were the predominant, although not abundant, morphotype observed on the surfaces of dried alder leaves prior to their incubation in the river. The pattern of colonisation of leaves incubated in the river was as follows:

Cocco-bacilli

These organisms were always the first organisms to colonise, replacing the yeast cells within the first week. Cocco-bacilli remained present throughout the leaf decay process although not always as the most

Figure 9: Typical yeast cells observed *in vivo* on dried alder leaves. Bar measurements a=5μm, b, c=10μm.


a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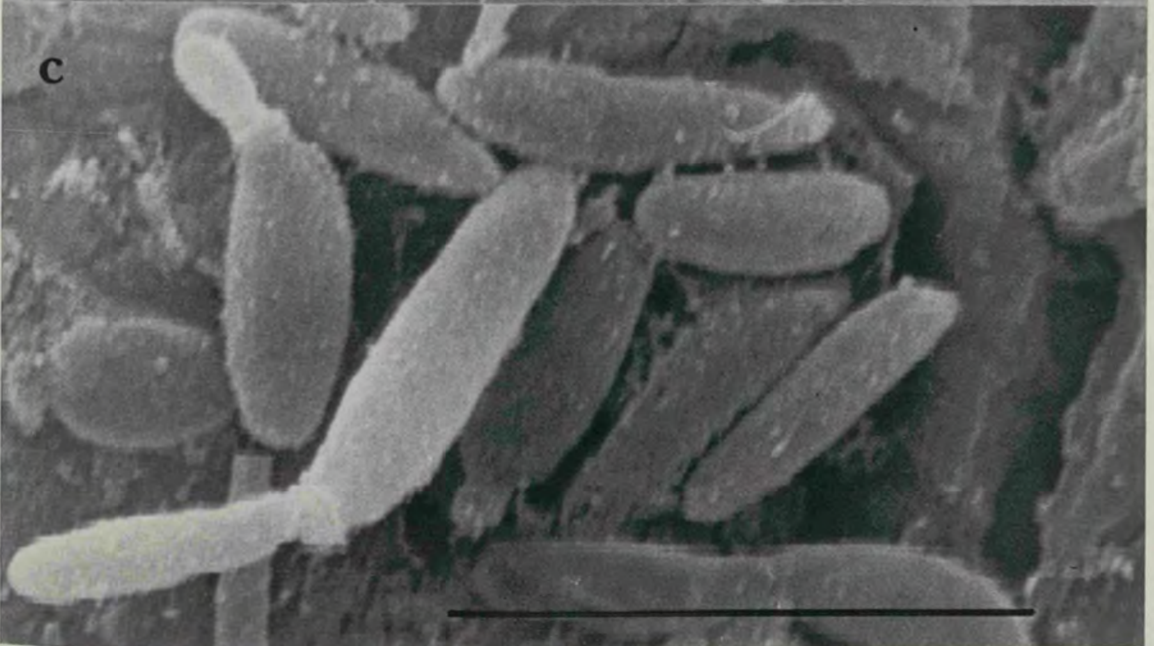

c

Figure 10: Typical cocco-bacilli observed *in vivo* on decaying alder leaves. Bar measurements a=5μm, b=5μm, c=10μm.

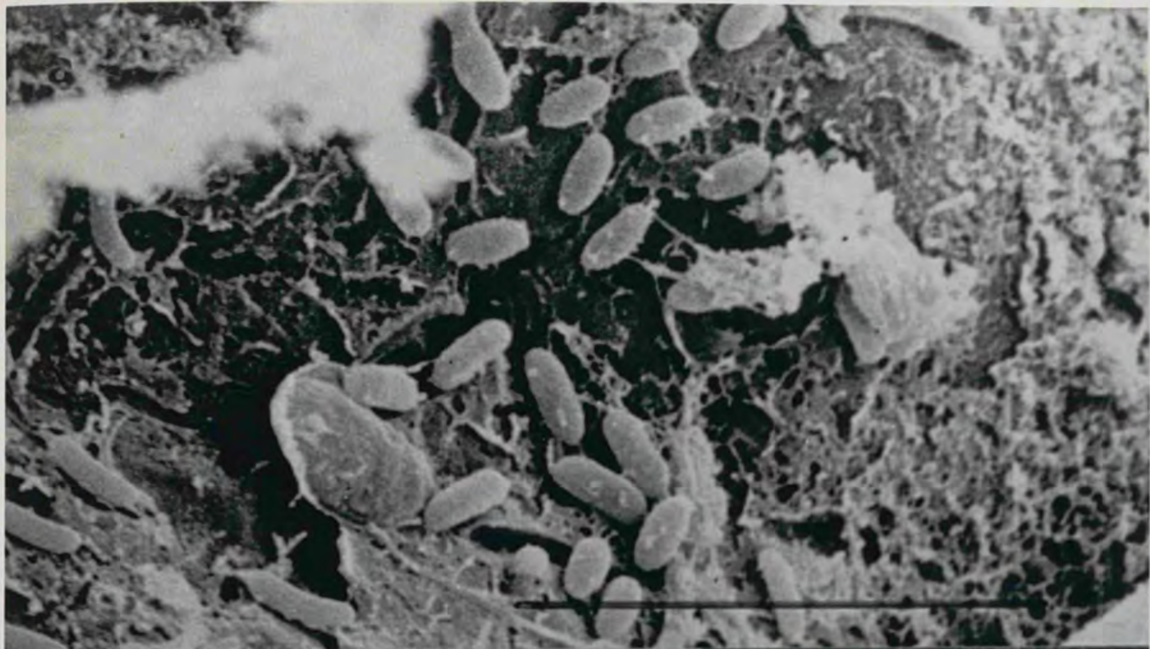
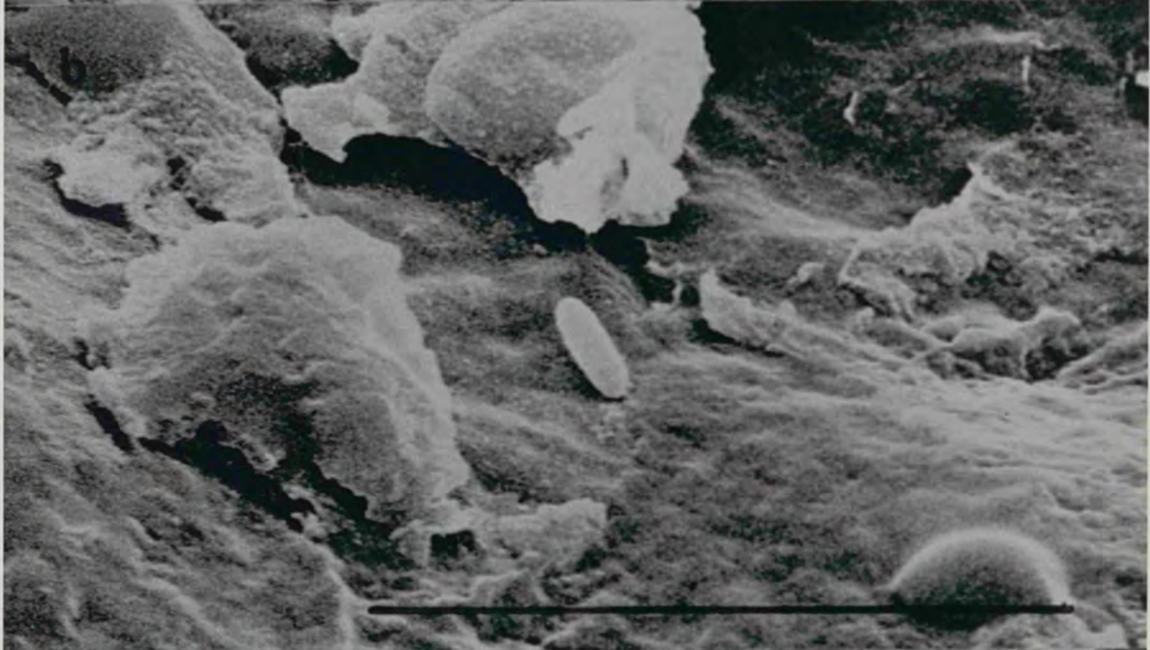
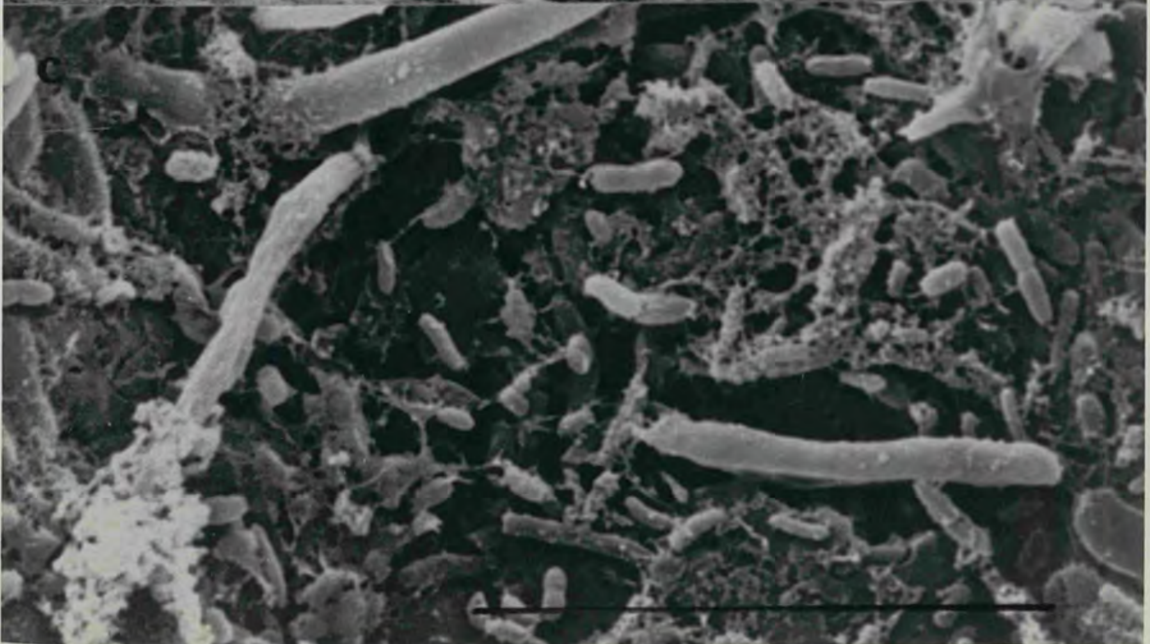
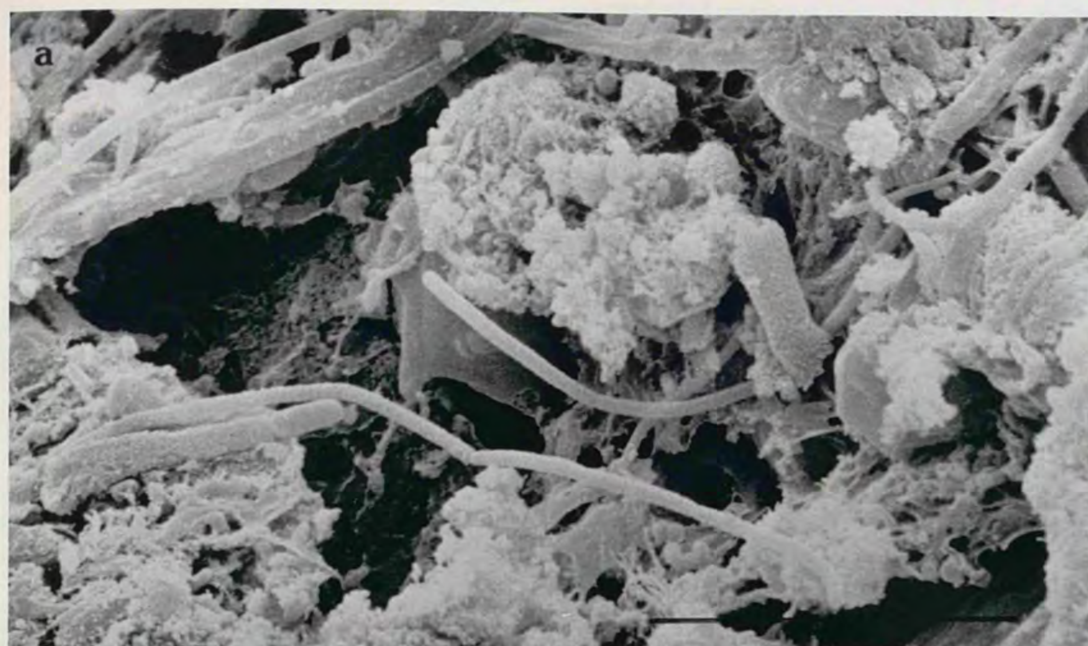




Figure 11: Typical imtermediate length bacilli observed *in vivo* on decaying alder leaves. Bar measurements a, b, c, d=10μm.

Figure 12: Typical flexuous bacilli observed *in vivo* on decaying alder leaves. Bar measurements a = 10 μm, b = 5 μm, c = 10 μm.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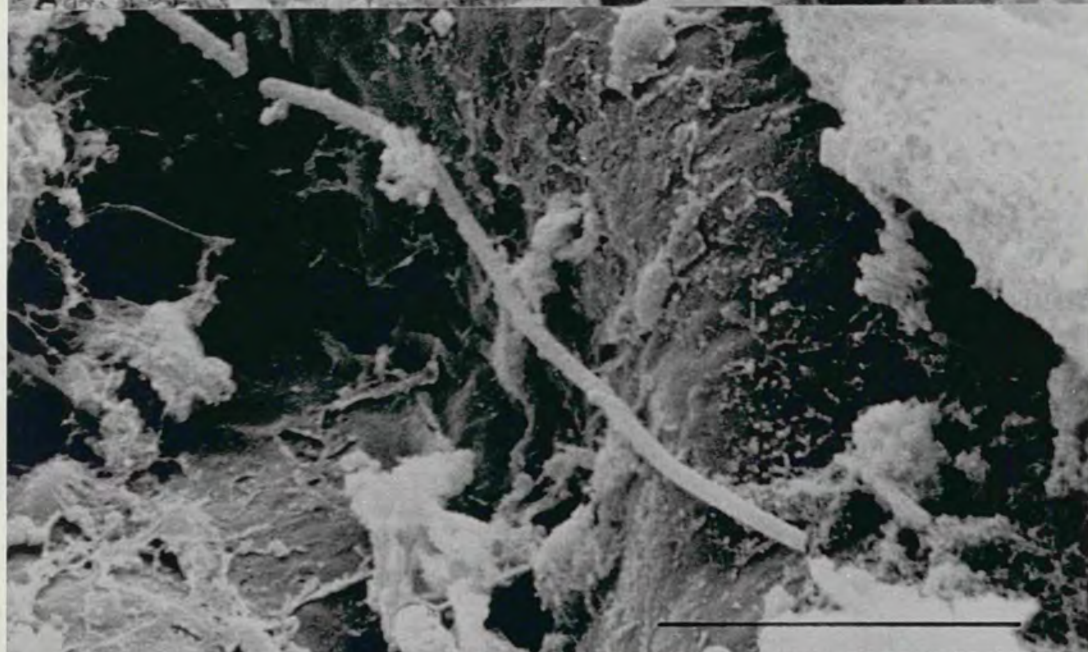


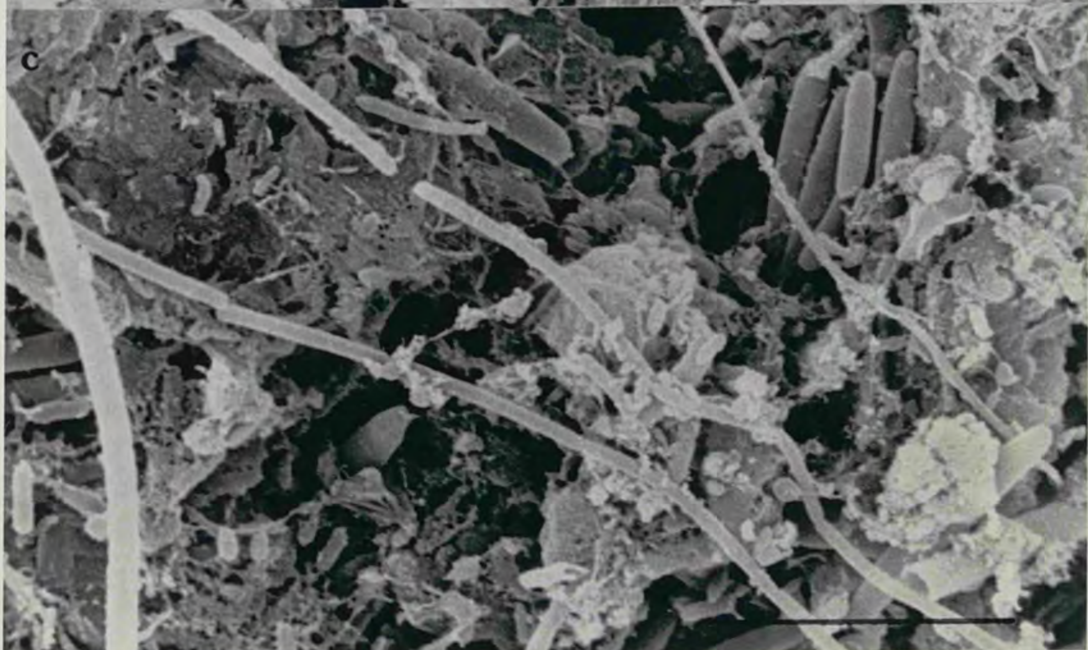
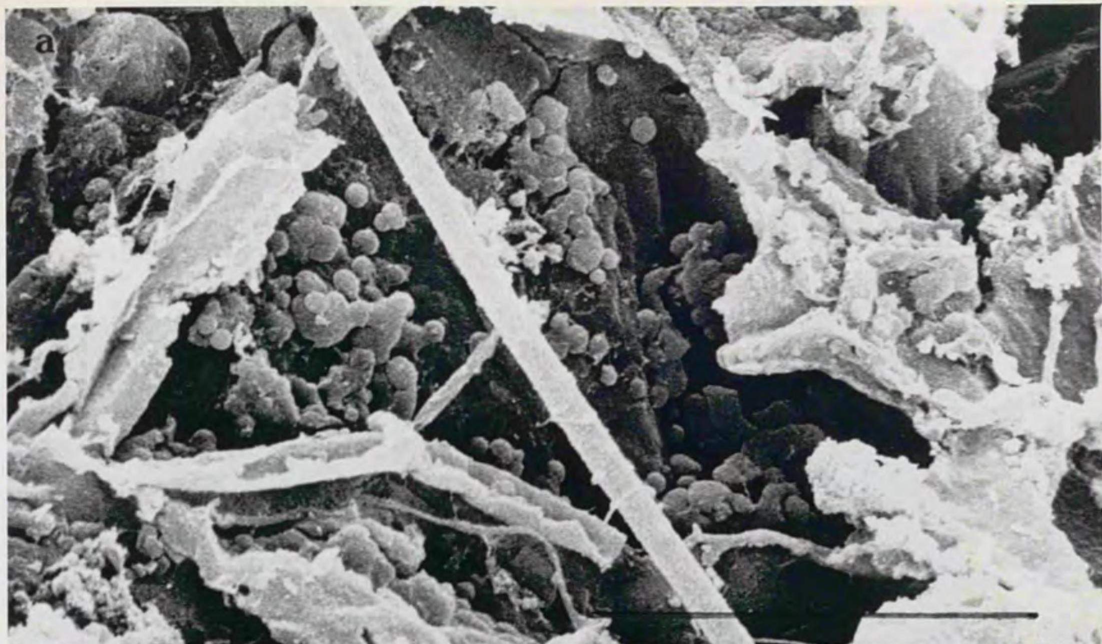

c

Figure 13: Typical cocci observed *in vivo* on decaying alder leaves. Bar measurements a=20μm, b=10μm, c=10μm.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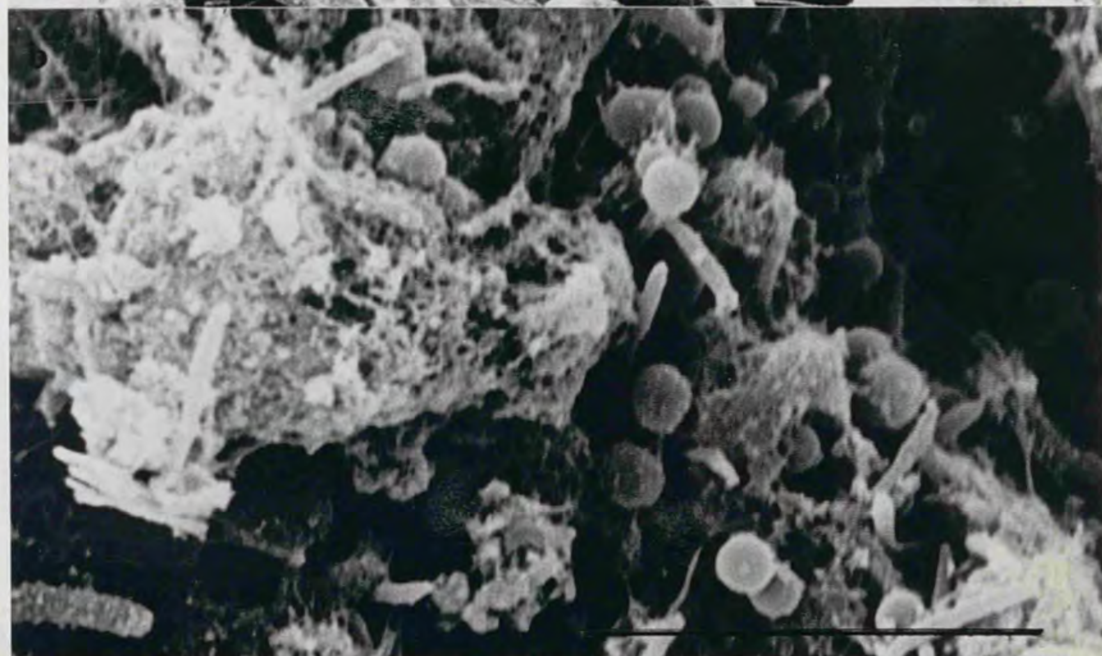


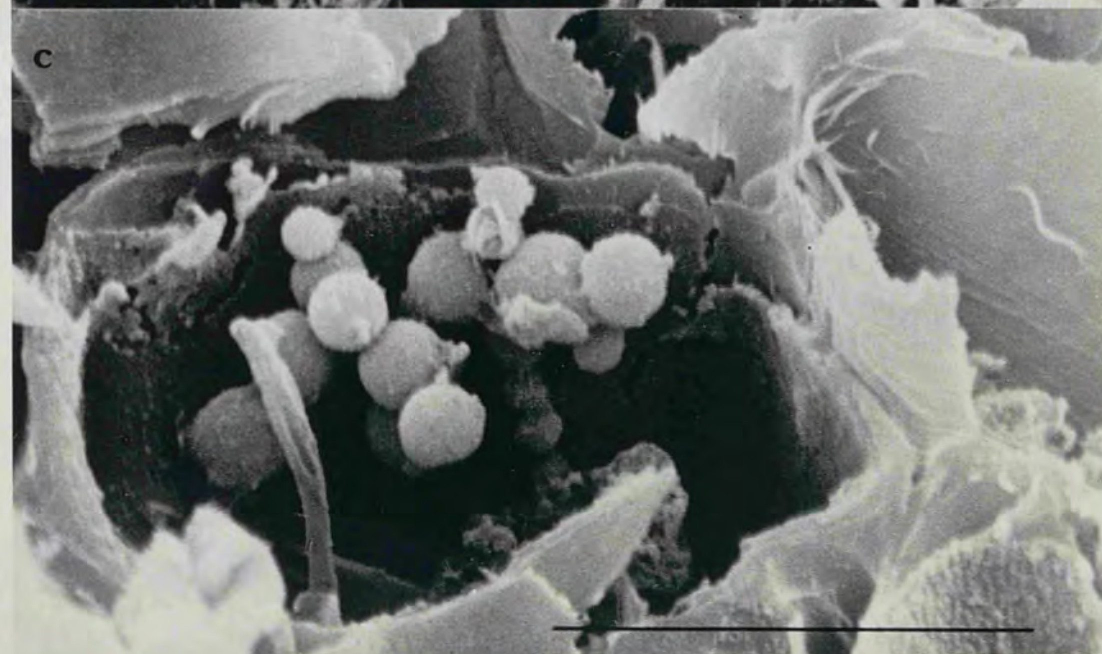
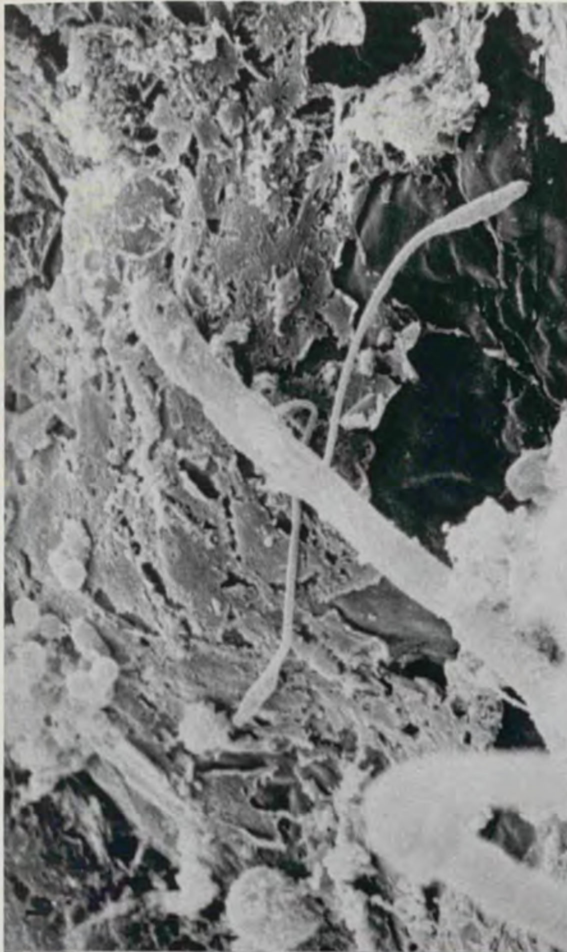
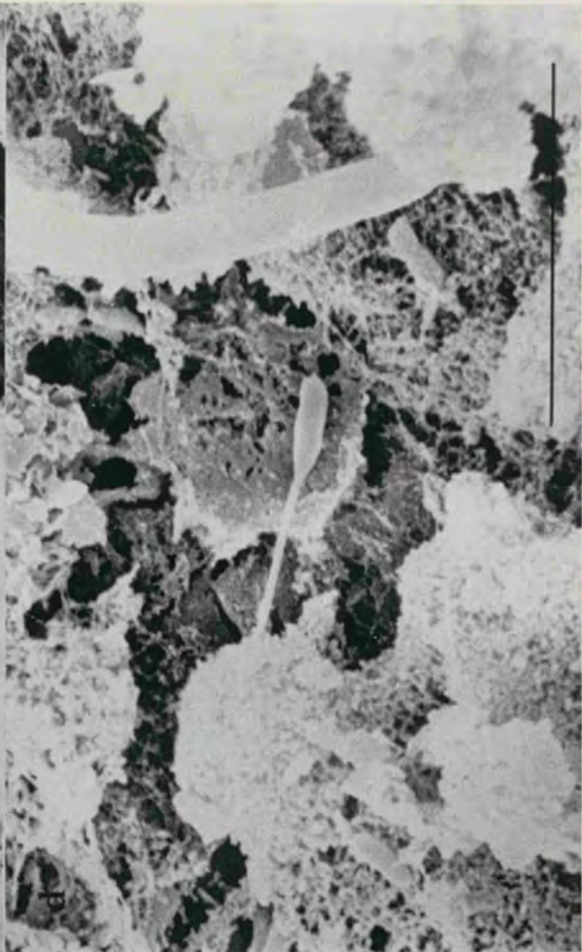
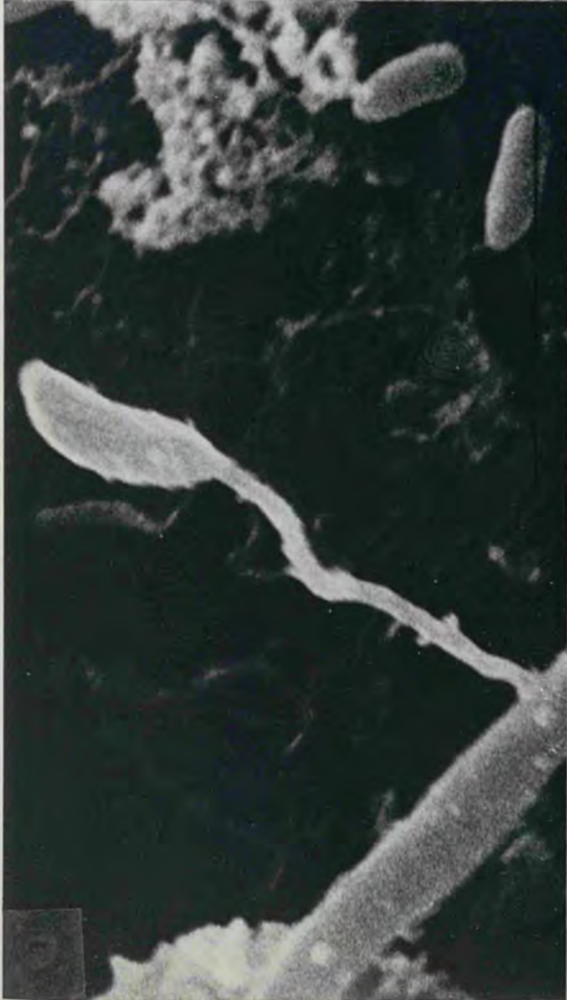

c

Figure 14: Typical prosthecate bacteria observed *in vivo* on decaying alder leaves. Bar measurements a=2μm, b=5μm, c=10μm, d=5μm.







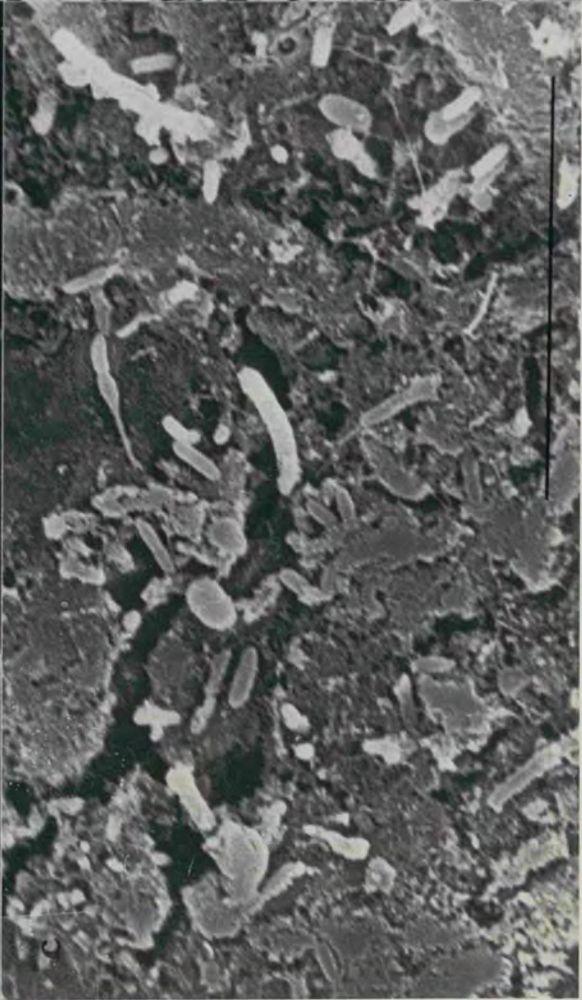
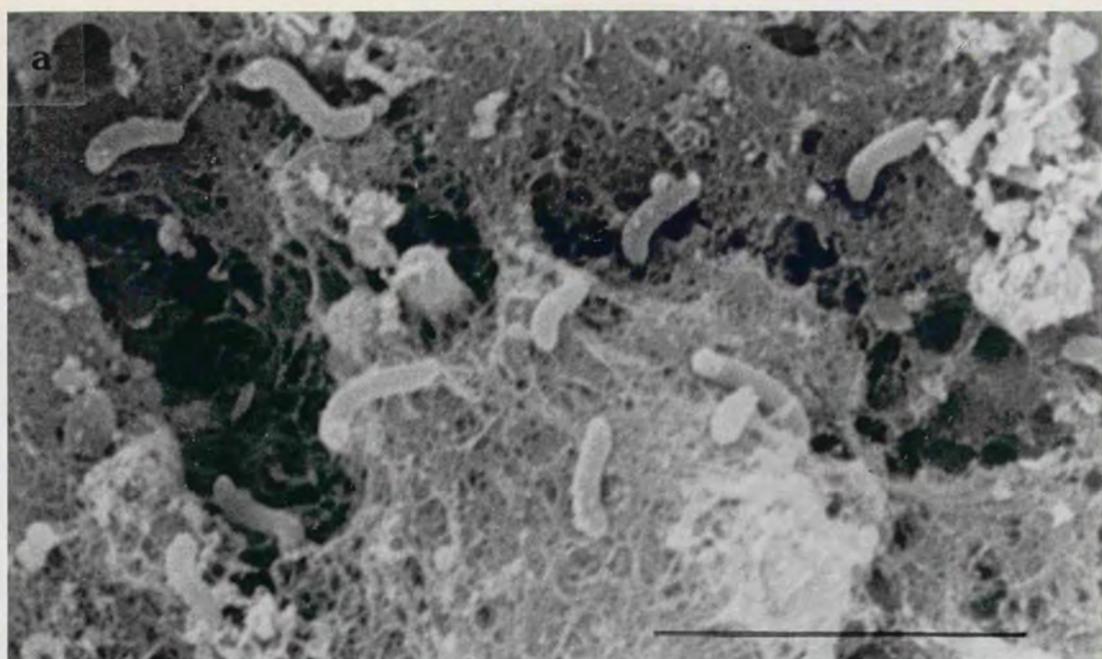


Figure 15: Typical S-shaped bacteria observed *in vivo* on decaying alder leaves. Bar measurements a, b, c=10μm.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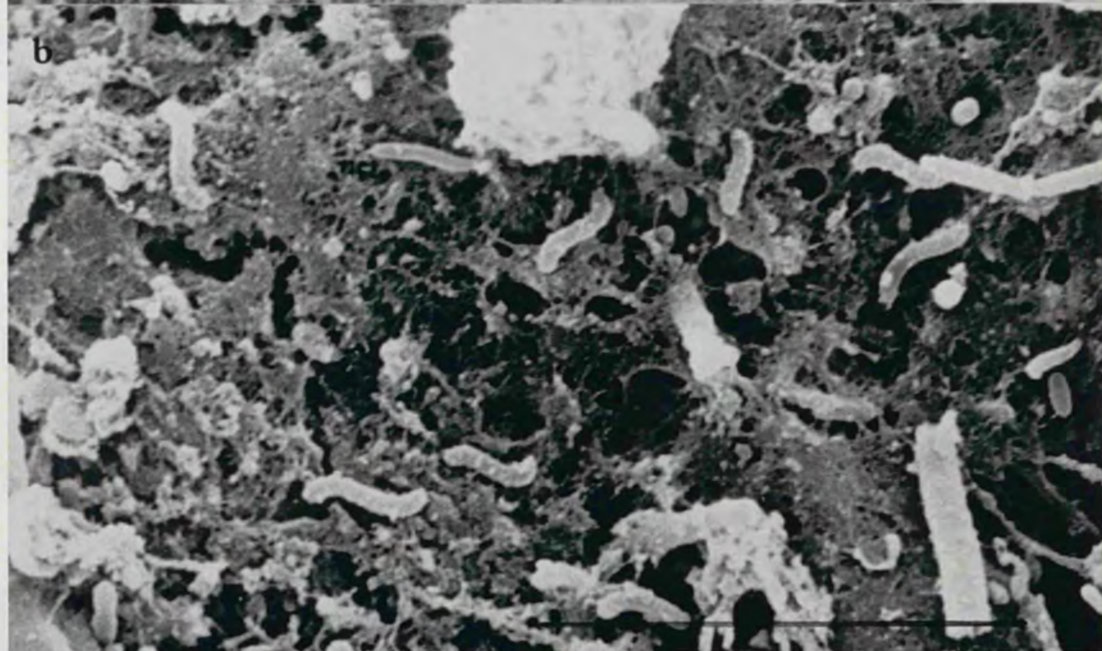

b

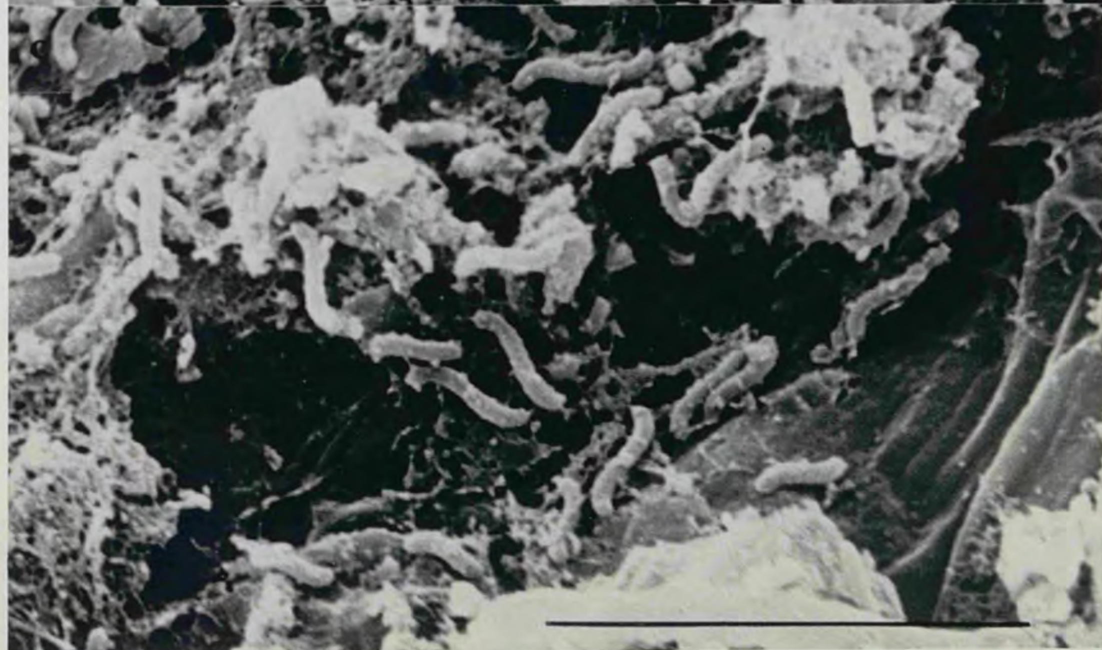
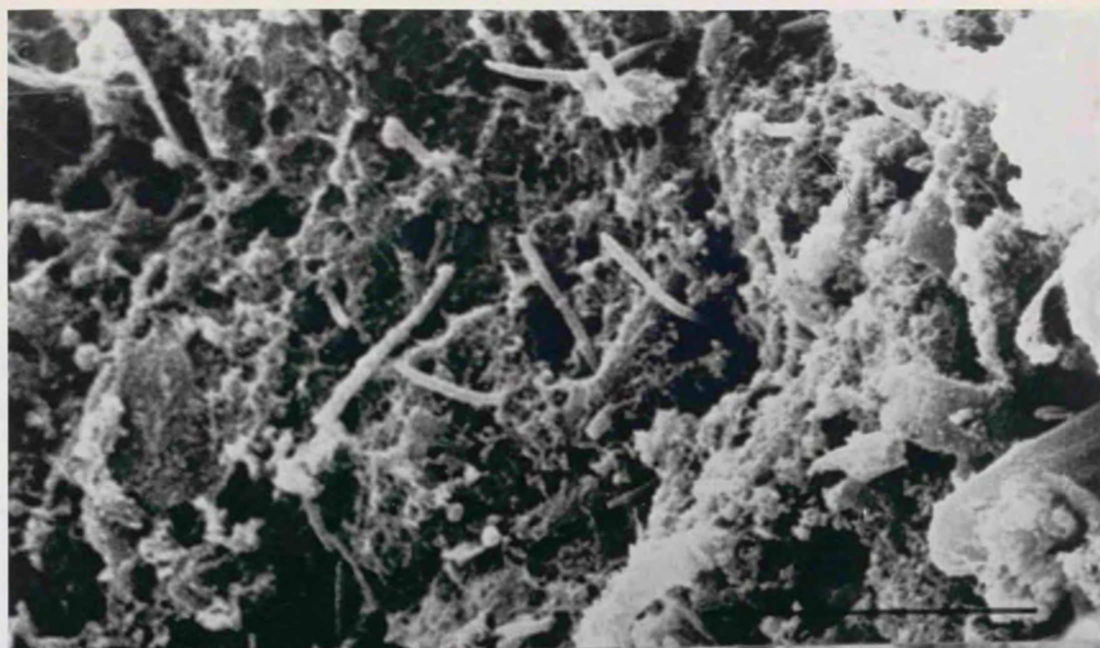
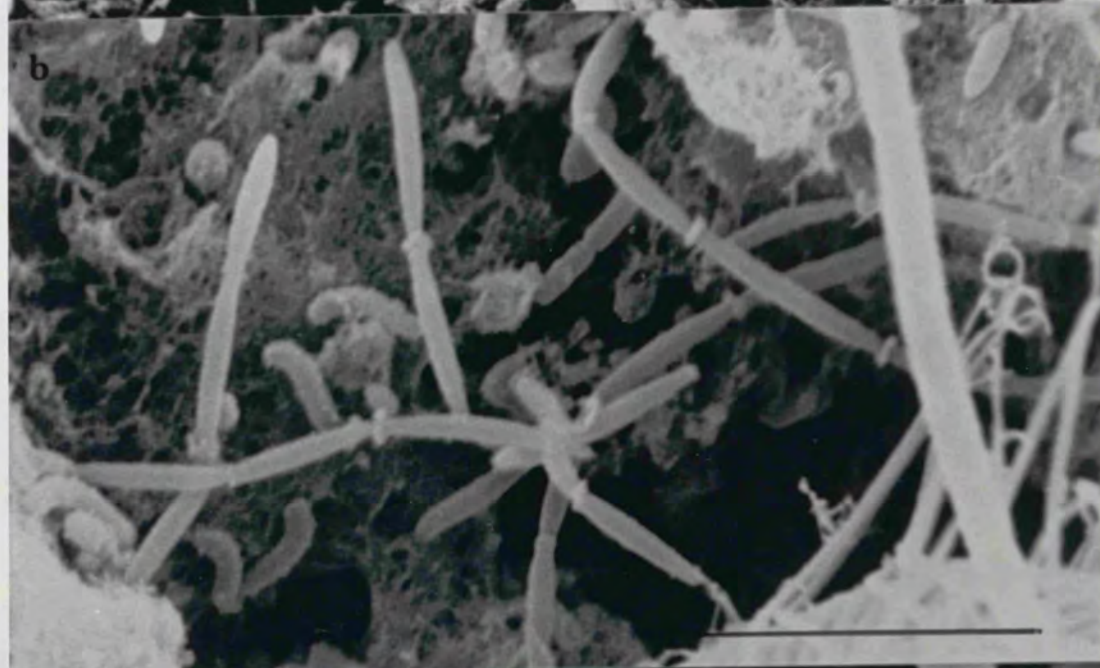

c

Figure 16: Typical chains of flexuous bacilli separated by "discs" observed *in vivo* on decaying alder leaves. Bar measurements a, b, c=10μm.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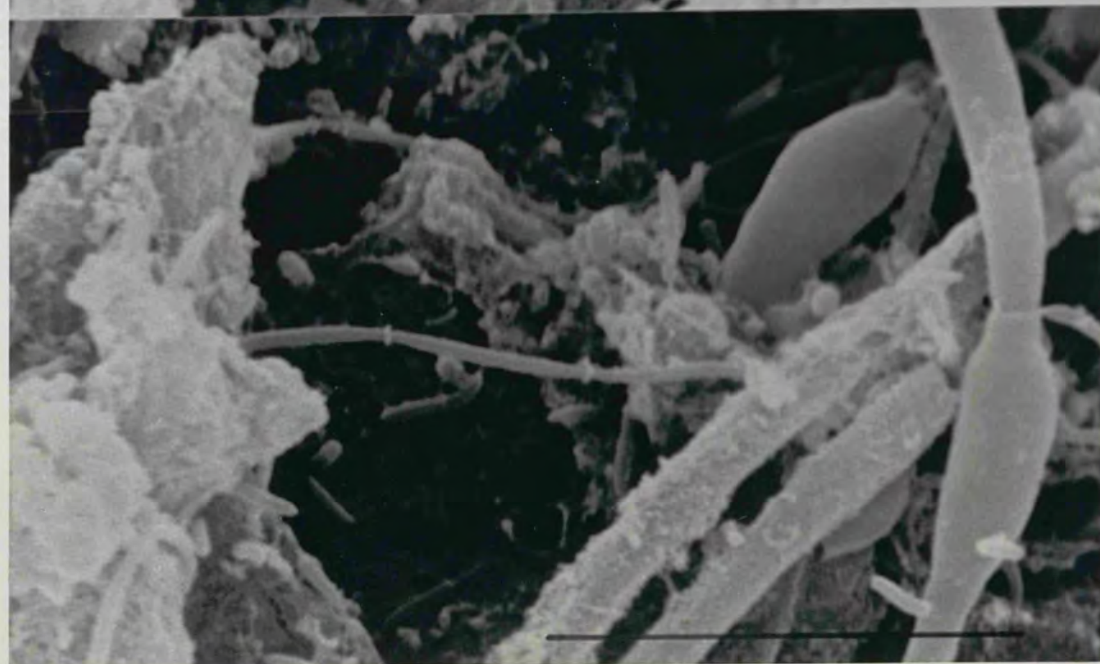
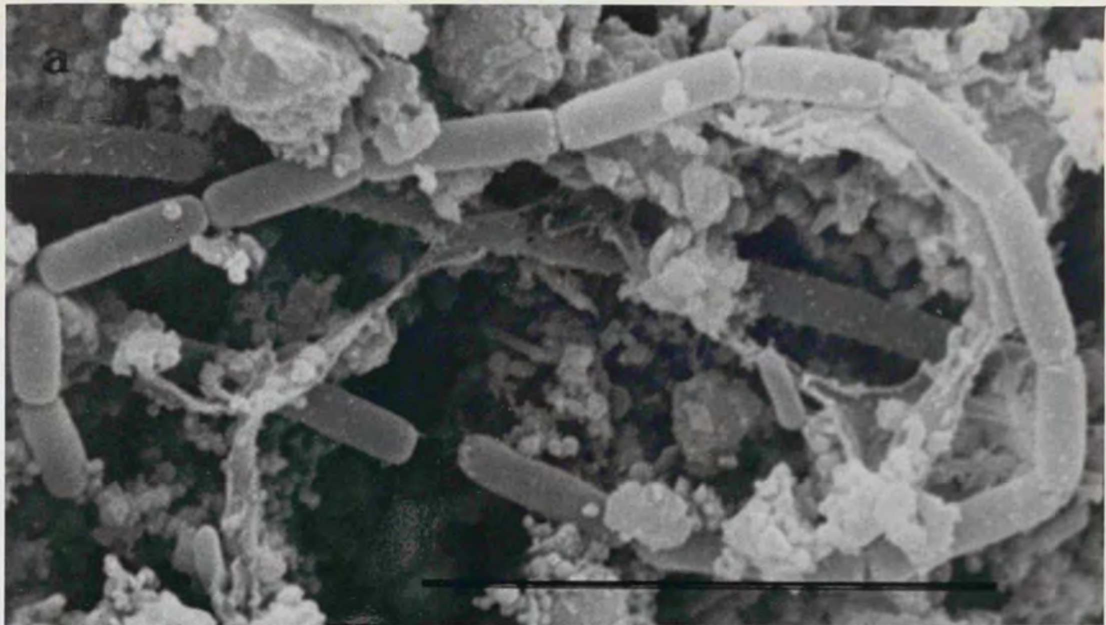


Figure 17: Typical brick-shaped bacilli observed *in vivo* on decaying alder leaves. Bar measurements a=10μm, b=2μm, c=5μm.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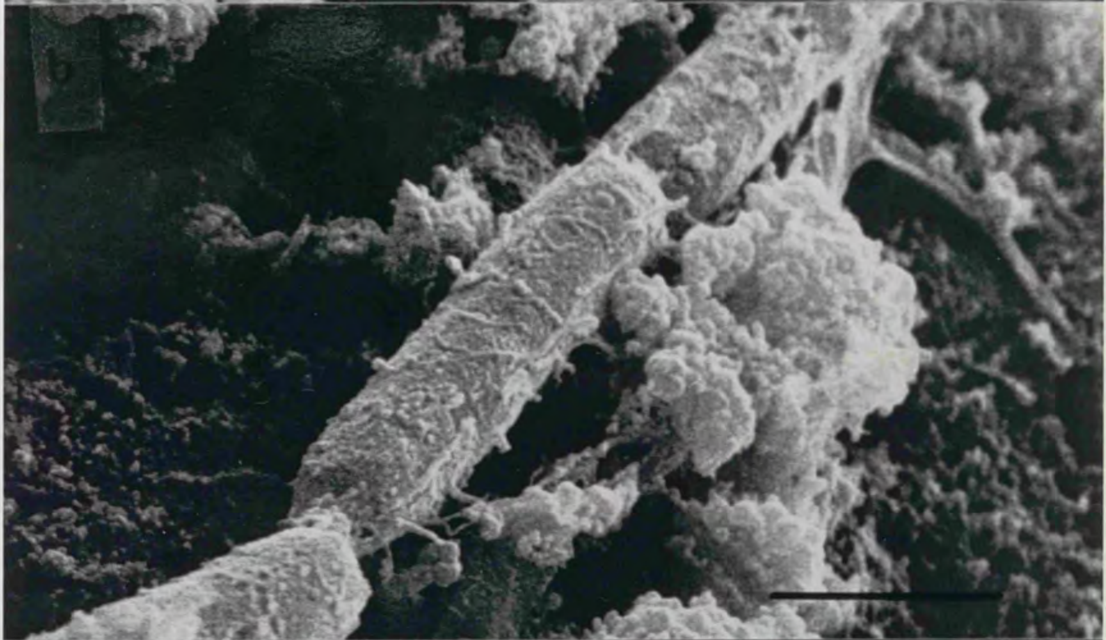

b

c

Colonisation pattern of alder leaves - key to symbols

Organism Morphologies

1.......yeast cells

2.......cocco-bacilli

3.......intermediate length bacilli

4.......flexuous bacilli

5.......cocci

6.......prosthecate bacteria

7.......s-shaped bacteria

8.......chains of flexuous bacilli separated by discs

9.......brick-shaped bacilli

Organism Abundance

+.......isolated

++......small groups, not abundant

+++.....abundant but not dominant

++++....abundant and dominant

Surface Debris

-.......absent

+.......present

Length of incubation time

h........hours

w........weeks

length of incubation	river temp °C	surface debri	Organism Morphology 1	2	3	4	5	6	7	8	9	special features
1W	12	-	++	++++			+					
2W	10	+		+++		+						
3W	10,5	+		+++		+		+				
4W	7	+		+++		+			++			
5W	7,5	+		++		++		++	+++	++		
6W	8,5	+				+++	+	++	++			

Table 8: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 1.

length of incubation	river temp °C	surface debri	Organism morphology 1	2	3	4	5	6	7	8	9	special features
24h	5	-	++++	++			++					
48h	6	-	+++	+++	++		++					cocco-bacilli associated with polysaccharide
72h	6	-	++	+++			++					"
96h	6	-	++	+++				+				"
												cocco-bacilli and intermediate length bacilli with polysaccharide
1W	5,5	-		++++	++			++			++	"
2W	5,5	+		+++	++	+	+	+++				
3W	6,5	+		+++	++	++++	+	+++				
4W	8,5	+		+++	++	++++		+++	++	++		
5W	10	+		+++		++			++	++		
6W	13,5	+		+++				+	+			

Table 9: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 2.

length of incubation	river temp °C	surface debri	Organism Morphology 1	2	3	4	5	6	7	8	9	special features
1W	17	+		+++	+	++	++	++	++		+	cocco-bacilli embedded in polysaccharide
2W	16	+		++++		++	++	++	++			
3W	20	+		+++				+	+	++		

Table 10: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 3.

length of incu-	river temp °C	surface debri	Organism morphology 1	2	3	4	5	6	7	8	9	special features
24h	16	-	++	+++			+	+				
48h	16	-	++	+++	+		+	+				cocco-bacilli associated with polysaccharide
72h	16	-	++	+++	++		+	+				"
96h	15	+	++	+++	++		+	+				"
1W	14	+		+++	++++			++				"
2W	13	+		+++	+++			++	+	+		"
3W	11	+		+++	+	+++		+		++		
4W	10	+		+++	+		+	+++		++		
5W	10	+		+++	+++		+	++			++	

Table 11: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 4

length of incu-	river temp °C	surface debri	Organism Morphology 1	2	3	4	5	6	7	8	9	special features
1h	11	-	++++	++								
2h	11	-	++++	++								
4h	11	-	++++	++								
6h	11	-	+++	+++								organisms always around stomata
24h	11	-	++	++				+				
48h	11	-	+	+++	++							
72h	11	+	+	++	+++							
1W	12	+		+++	+++		++	++				cocco-bacilli associated with polysaccharide
2W	12	+		+++	+++			++	++			"
3W	13,5	+		++	++	++		+++			++	"
4W	13	+		++	++	++		++	++			
5W	13	+		++					++		++	

Table 12: Colonisation pattern of alder leaves incubated in freshwater. Experimental run number 5.

abundant morphotype. Initial colonisation was by isolated organisms progressing to groups of less than 10 individuals. The organisms were sometimes observed in pairs. The organisms were usually spatially separated within the small groups, but appeared closer together or in direct contact with one another as the sizes of the groups increased with increased length of incubation time. Cocco-bacilli were often observed embedded in polysaccharide particularly during the first 3 weeks of colonisation.

Intermediate length bacilli

This morphotype followed the cocco-bacilli, but always appeared to be less abundant. These organisms were also occasionally observed embedded in polysaccharide.

Flexuous forms

This morphotype followed the intermediate length bacilli and tended to become more abundant after 2 to 3 weeks and persist thereafter.

Cocci

Cocci were always present but in low numbers.

Prosthecate organisms

This morphotype appeared after 24 hours incubation in run number 4, but only in isolation. They generally appeared in greater abundance after 1-2 weeks incubation.

S-shaped organisms

S-shaped organisms appeared after 3 weeks incubation or later. They tended to become less plentiful towards the end of the decay period.

Chains of flexuous bacilli separated by "discs"

These organisms appeared after 2 weeks incubation or later. They were observed in runs 1-4, but not 5.

Brick-shaped bacilli

This morphotype appeared occassionally.

A distinct pattern of colonisation was observed during the first 3 weeks of runs 1, 2, 4 and 5. Leaf surfaces tended to accumulate layers of debris and this obstructed view of the microorganisms towards the later stages of decay. It was therefore unclear whether or not there was a succession of microorganisms during these stages. Enumeration of morphotypes using SEM was also considered impractical for this reason.

Run number 3 was excluded from assessment of succession because of the the very short decay period encountered in this run. In view of this, weekly samplings would have been too infrequent to detect a pattern of colonisation.

Runs 2, 3 and 5 were conducted during periods when the river temperature was increasing and 1 and 4 when it

was decreasing (tables 8-12) and show that decay rate was faster when the average run temperature (table 13) was higher. No marked difference between morphotypes was observed between experimental runs which tends to suggest that temperature did not affect the type of organism which colonised, but possibly the metabolic activity of the organisms.

Leaf packs were sampled more frequently during the first week of incubation (tables 8-12) during some runs to assess whether a noticeable change in bacterial morphotype occurred in response to leaching of soluble low molecular weight compounds from the leaves. Gradual and progressive changes in abundance of some morphotypes was observed during the first 7 days of incubation although it was unclear whether these changes were solely in response to leaching, temperature, physico-chemical conditions or a combination of these factors.

Run no	Average temperature °C
1	9,25
2	7,25
3	17,7
4	13,4
5	11,7

Table 13: Average water temperatures during incubation periods of alder leaves in the River Bourne.

Preparation of samples for SEM observation was done using a variety of techniques. Samples from runs 1 and 2 were fixed in 2,5% glutaraldehyde, 3 and 4 in osmium tetroxide vapour and 5 in 2,5% osmium tetroxide. Fixation in osmium tetroxide was found to produce better quality specimens for SEM and these samples could be successfully focused at higher magnifications than those fixed in glutaraldehyde. There was no identifiable difference between samples fixed in osmium tetroxide vapour for 20 minutes and those fixed in 2,5% osmium tetroxide for 4 hours. There was also no observable difference between samples dehydrated through a graded acetone series and those processed in the VDD apparatus. VDD was, however, a more convenient method, not requiring regular transfers of specimens to different acetone concentrations to be made. VDD was

also a more convenient method for dehydration of a number of different specimens.

2. Enumeration of Bacteria colonising decaying alder leaves

Bacteria on the surfaces of alder leaves were enumerated during the course of experimental runs 2 and 5. The number of bacteria colonising sterile alder leaf discs *in vitro* was also determined. The organisms were stained with acridine orange and viewed under ultraviolet light at 500 or 1000 x magnification. Bacteria within a predetermined area of a grid-marked eye-piece, previously calibrated using a stage micrometer, were counted, 5 fields on each of 5 discs for each daily or weekly sample. The total number of bacteria/cm^2 was calculated for each field counted and the results were analysed using a one way analysis of variance (Minitab) and the confidence limit, using the student's T test, computed (figs. 18-20 and Tables 1-3 in Appendix).

These results are presented graphically in figs. 21-23, correctly scaled.

Leaf discs which had been stained with acridine orange were mounted on microscope slides and covered with a coverslip in preparation for viewing. The uneven surface and rigidity of the leaf discs did not allow for uniform contact between the leaf disc and coverslip

```
ANALYSIS OF VARIANCE

SOURCE     DF        SS        MS        F
FACTOR      8 2.460E+16 3.076E+15    22.55
ERROR     201 2.741E+16 1.364E+14
TOTAL     209 5.202E+16

                                    INDIVIDUAL 95 PCT CI'S FOR MEAN
                                    BASED ON POOLED STDEV
LEVEL       N      MEAN     STDEV   ----+---------+---------+---------+--
Day1       25  17111600   9473688                (---*---)
Day2       25  24595200  12787382                       (--*---)
Day3       25  28398000  12722749                          (---*---)
Day4       25  27956000  13331965                         (---*---)
Week1      25  33535600  16676140                              (---*---)
Week2      25  16618800  16408100                (---*---)
Week3      25   3479200    835713      (---*---)
Week4      25   3981600    920827      (---*---)
Week5      10   3334000   1072818    (-----*-----)
                                    ----+---------+---------+---------+--
POOLED STDEV = 11678146                 0  12000000  24000000  36000000
```

Figure 18: Analysis of variance between number of bacteria/cm^2 on degrading alder leaves in experimental run number 2.

```
ANALYSIS OF VARIANCE

SOURCE      DF        SS        MS         F
FACTOR       9 2.563E+15 2.847E+14    126.93
ERROR      240 5.384E+14 2.243E+12
TOTAL      249 3.101E+15
                                  INDIVIDUAL 95 PCT CI'S FOR MEAN
                                  BASED ON POOLED STDEV
LEVEL        N      MEAN     STDEV  ------+---------+---------+---------+
day1        25   1835600    446067  (-*-)
day2        25   2725200    826081     (-*-)
day3        25   2832800    728792     (-*-)
Week1       25   2713600    400602     (-*-)
Week2       25   2552400    470295     (-*)
Week3       25   3751600    598545         (-*)
Week4       25   6583600   2434394                  (-*-)
Week5       25   9509200   2072362                            (-*-)
Week6       25  10138000   2236569                              (-*-)
Week7       25   9593600   2248683                            (-*-)
                                    ------+---------+---------+---------+
POOLED STDEV =   1497739          3000000   6000000   9000000  12000000
```

Figure 19: Analysis of variance between number of $bacteria/cm^2$ on degrading alder leaves in experimental run number 5.

```
ANALYSIS OF VARIANCE

SOURCE      DF        SS        MS         F
FACTOR       7 9.663E+16 1.380E+16     38.35
ERROR      181 6.516E+16 3.600E+14
TOTAL      188 1.618E+17


                                     INDIVIDUAL 95 PCT CI'S FOR MEAN
                                     BASED ON POOLED STDEV
LEVEL       N      MEAN     STDEV    ---+---------+---------+---------+---
Day1       19  55889472  36459124                          (--*---)
Day2       25  20864000  14015089            (--*--)
Day3       25  36053600  20549976                  (--*--)
Day4       25  44988000  20507546                      (--*--)
Week1      20  61965000  29127350                            (---*--)
Week2      25   2069600   2356612     (--*--)
Week3      25    838080    246825    (--*--)
Week4      25   4535200   1158840      (--*--)
                                     ---+---------+---------+---------+---
POOLED STDEV = 18973122                0  24000000  48000000  72000000
```

Figure 20: Analysis of variance between number of bacteria/cm^2 of alder leaf discs incubated _in vitro_ with river water in a closed system continuous flow fermentor.

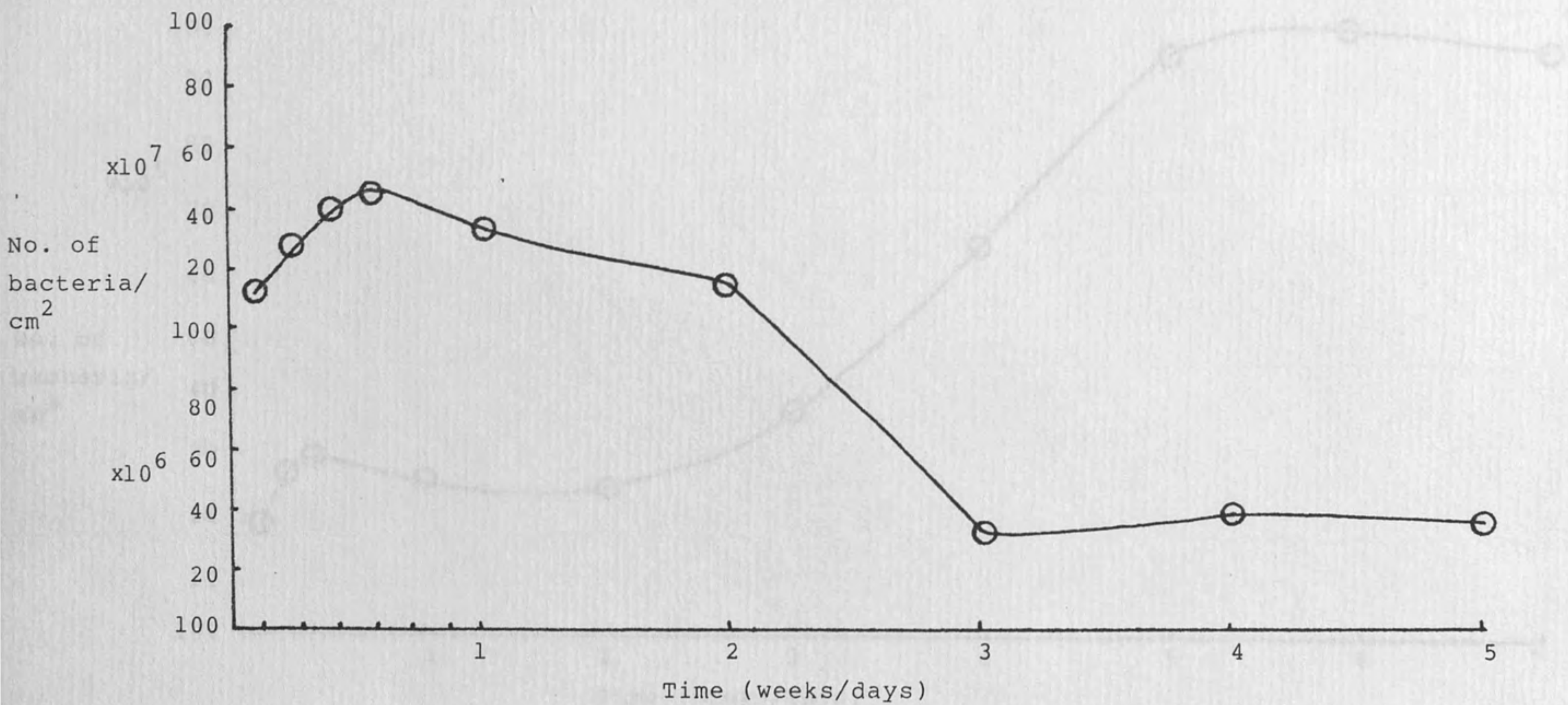


Figure 21: Change in number of bacteria/cm^2 of alder leaf surface during experimental run number 2.

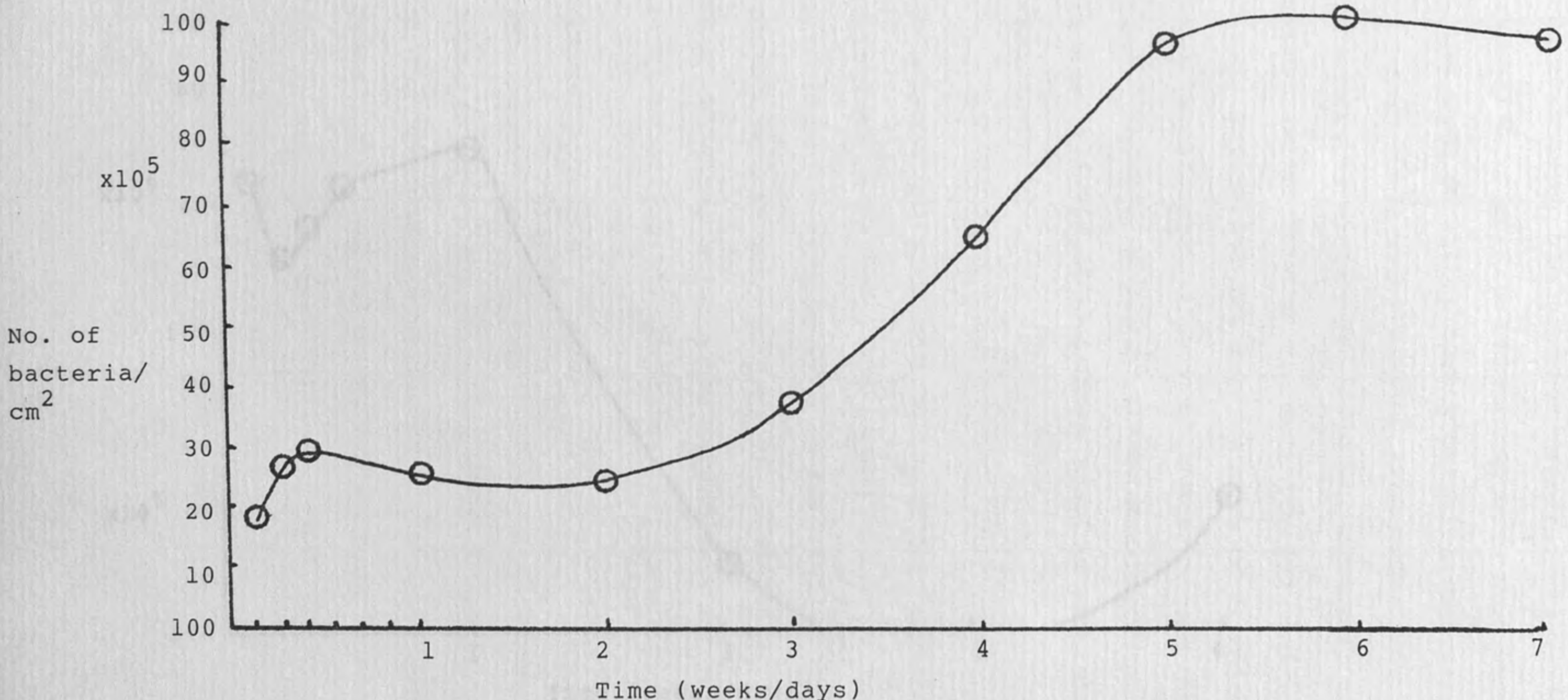


Figure 22: Change in number of bacteria/cm^2 of alder leaf surface during experimental run number 5.

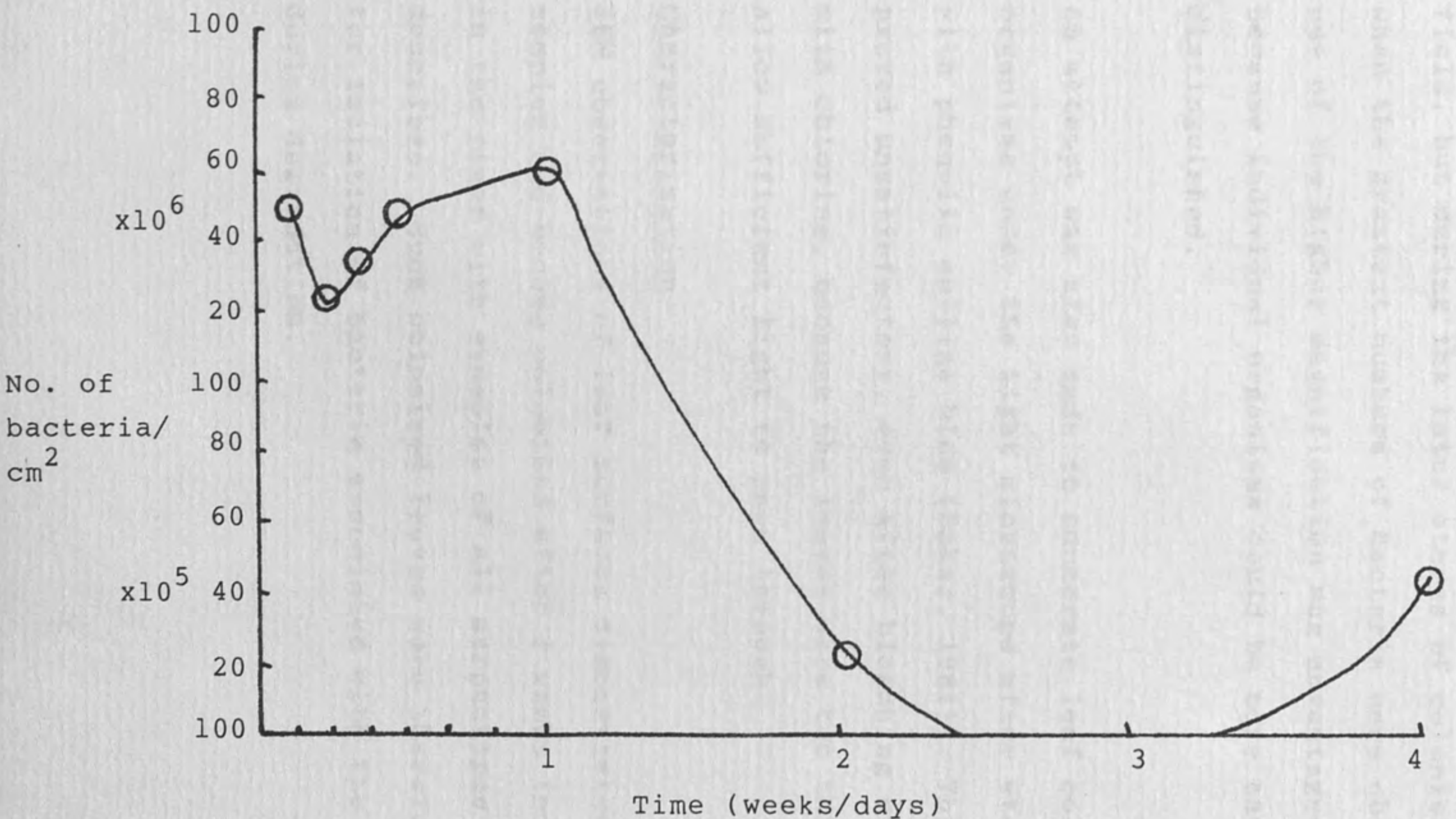


Figure 23: Change in number of bacteria/cm^2 of alder leaf discs incubated _in vitro_ with river water in a closed system continuous flow fermentor.

and a drop of sterile water was therefore added in order to assist in maintaining contact. Immersion oil was also used, but superior results were obtained using water. Leaf surface colonising organisms were enumerated at either 1000 x magnification (run number 2) or at 500 x magnification (run number 5). Focusing on an entire microscope field was difficult at high magnification because of the variation in depth of field, but during the later stages of colonisation, when the greatest numbers of bacteria were observed, use of the higher magnification was advantageous because individual organisms could be more easily distinguished.

An attempt was also made to enumerate leaf colonising organisms under the light microscope after staining with phenolic aniline blue (Baker, 1981). This method proved unsatisfactory, even after bleaching of leaves with chlorine, because the leaves were too thick to allow sufficient light to pass through.

3. Characterisation

SEM observations of leaf surfaces demonstrated that samples had become colonised after 3 weeks incubation in the river with examples of all morphotypes described. Such colonised leaves were therefore used for isolation of bacteria associated with the leaves during degradation.

Keys to tables 14-19

* cultures which died during the course of processing

1 = lactose fermentation

2 = citrate utilization

3 = transmission electron microscopic viewing of flagellar number and insertion

4 = gelatin liquefaction

5 = digestion of filter paper

6 = growth at 42°C

7 = sensitivity to polymyxin B

8 = Hugh and Liefson's O/F test

O = oxidative

F = fermentative

9 = bacteriolytic activity

10 = carbohydrate fermentation (Dye's method)

a = mannose

b = galactose

c = cellobiose

d = adonitol

e = inositol

API = tested on API 20NE strip

FAP = analysis by fatty acid profiling

Isolate number / Characteristic	1	2	3	4	5
Medium of isolation	←Nutrient Agar→				
Colony pigment and morphology	yellow				yellow
Gram stain	-	-	-	-	-
Morphology	cocci	rods	rods	rods	rod
Catalase	+	+	+	+	+
Oxidase	-	+	+	+	+
Motility	-	+	+	+	-
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	+	-	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	6*	7	8	9	10
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology		yellow			yellow
Gram stain		-	-	-	-
Morphology		rod	rod	rods	rods
Catalase		+	+	+	+
Oxidase		+	+	+	+
Motility		+	+	+	+
Glucose fermentation		-	-	-	
Fluorescent pigment production		+	+	+	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	11	12*	13*	14	15
Medium of isolation	←		Nutrient Agar		→
Colony pigment and morphology	yellow			yellow	purple rubbery
Gram stain	+	-		-	-
Morphology	rods			rods	rods
Catalase	+			+	+
Oxidase	+			+	+
Motility	+			+	+
Glucose fermentation	acid			-	-
Fluorescent pigment production	-	-		+	-
Growth in anaerobic jar	-	-		-	-
Indole production					
Additional tests					FAP

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	16*	17*	18	19	20
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology	turned agar brown			yellow	
Gram stain	-		-	-	-
Morphology	rod		rod	rod	rod
Catalase	-		+	+	+
Oxidase	+		+	+	+
Motility	-		+	+	+
Glucose fermentation	-		-	-	-
Fluorescent pigment production	-		+	+	+
Growth in anaerobic jar	-		-	-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	21	22	23	24*	25
Medium of isolation	←	Nutrient Agar			→
Colony pigment and morphology					yellow
Gram stain	-	-	-		-
Morphology	rod	rod	rod		rod
Catalase	+	+	+		+
Oxidase	+	+	+		+
Motility	+	+	+		+
Glucose fermentation	acid	acid	acid		acid
Fluorescent pigment production	+	+	+		+
Growth in anaerobic jar	-	-	-		-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	26	27	28	29*	30
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology		yellow			
Gram stain	-	-	+		-
Morphology	rod	rod	Cocci		rods
Catalase	+	+	-		+
Oxidase	+	+	-		+
Motility	+	+	-		+
Glucose fermentation	acid	acid	-		-
Fluorescent pigment production	+	+	-		+
Growth in anaerobic jar	-	-	-		-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	31	32	33	34	35
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation		acid	acid	acid	acid
Fluorescent pigment production	+	+	+	+	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	36	37	38	39	40
Medium of isolation	←	Nutrient Agar			→
Colony pigment and morphology		yellow	yellow	yellow	
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	acid	acid	-	-	-
Fluorescent pigment production	-	+	+	+	+
Growth in anaerobic jar	+	-	-	-	-
Indole production					
Additional tests		API	API	API	

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	41	42	43	44	45
Medium of isolation	←	Nutrient Agar			→
Colony pigment and morphology	mucoid		mucoid	mucoid	
Gram stain	-	-	-	-	-
Morphology	rods	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	-	+	-	+	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests				API	API

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	46	47	48	49	50
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology	mucoid	yellow		yellow	yellow
Gram stain	-	-	-	-	+
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	+	-	-
Growth in anaerobic jar	-	-	-		-
Indole production				-	
Additional tests		API	API	API	

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	51*	52	53	54	55
Medium of isolation	←	Nutrient Agar			→
Colony pigment and morphology	yellow				
Gram stain	+	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase		+	+	+	+
Oxidase		+	+	+	+
Motility		+	+	+	+
Glucose fermentation		-	-	-	-
Fluorescent pigment production		+	+	+	-
Growth in anaerobic jar		-	-	-	-
Indole production					
Additional tests		API	API	API	API

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	56	57	58	59	60*
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology					
Gram stain	-	-	-	-	
Morphology	rod	rod	rod	rod	
Catalase	+	+	+	+	
Oxidase	+	+	+	+	
Motility	+	+	+	+	
Glucose fermentation	-	-	-	-	
Fluorescent pigment production	-	+	+	-	
Growth in anaerobic jar	-	-	-	-	
Indole production					
Additional tests	API				

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	61	62	63	64	65
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	-	+	+	+
Growth in anaerobic jar	-	-	-	-	+
Indole production					
Additional tests	API	API			API

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	66	67	68*	69	70
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology			yellow		yellow
Gram stain	-	+	-	-	-
Morphology	rod	cocci	rod	rod	rod
Catalase	+	-	-	+	+
Oxidase	+	+	-	+	+
Motility	+	-	-	+	+
Glucose fermentation	+	-	-	-	-
Fluorescent pigment production	+	-	-	+	+
Growth in anaerobic jar	+	+	+	-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	71	72	73	74	75
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	-	+	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests	API		API		

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	76*	77	78*	79	80
Medium of isolation	←	Nutrient Agar			→
Colony pigment and morphology				yellow	
Gram stain		-		-	-
Morphology		rod		rod	rod
Catalase		+		+	+
Oxidase		+		+	+
Motility		+		+	+
Glucose fermentation		-		-	-
Fluorescent pigment production		-		-	+
Growth in anaerobic jar		-		-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	81	82	83	84	85
Medium of isolation	←	Nutrient Agar			→
Colony pigment and morphology	yellow				
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	+	+	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests	API				

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	86	87*	88	89	90
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology	yellow				
Gram stain	-		-	-	-
Morphology	rod		rod	rod	rod
Catalase	+		+	+	+
Oxidase	+		+	+	+
Motility	+		+	+	+
Glucose fermentation	-		-	-	-
Fluorescent pigment production	+		+	+	+
Growth in anaerobic jar	-		-	-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	91	92	93	94	95
Medium of isolation	←	Nutrient Agar			→
Colony pigment and morphology	yellow	yellow		yellow	
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	+	-	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

(continued ...)

Isolate number / Characteristic	96	97	98	99*	100
Medium of isolation	← Nutrient Agar →				
Colony pigment and morphology					mucoid
Gram stain	-	-	-		-
Morphology	rod	rod	rod		rod
Catalase	+	+	+		+
Oxidase	+	+	+		+
Motility	+	+	+		+
Glucose fermentation	-	-	-		-
Fluorescent pigment production	+	+	+		+
Growth in anaerobic jar	-	-	-		-
Indole production					
Additional tests					

Table 14: Morphological and biochemical characteristics of randomly selected isolates removed from the surfaces of colonised alder leaves and cultured on NA

Isolate number / Characteristic	101	102	103	104	105
Medium of isolation	NA	OA	NA	TA	TA
Colony pigment and morphology					
Gram stain	-	-	-	+	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	-	+
Motility	+	+	+	-	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	+	-	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests					

Table 15: Morphological and biochemical characteristics obtained by enrichment of isolates from colonised alder leaves with homogenised alder leaves in a mineral medium. Washings were cultured on selective agars.

(continued ...)

Isolate number / Characteristic	106	107	108*	109	110
Medium of isolation	TA	OA	NA	PSS	PSS
Colony pigment and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	-	+	+
Motility	+	+	-	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	-	+	+
Growth in anaerobic jar	-	-		-	-
Indole production					
Additional tests					

Table 15: Morphological and biochemical characteristics obtained by enrichment of isolates from colonised alder leaves with homogenised alder leaves in a mineral medium. Washings were cultured on selective agars.

(continued ...)

Isolate number / Characteristic	111	112	113	114	115
Medium of isolation	PSS	TA	NA	OA	NA
Colony pigment and morphology			turned medium brown		
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	-	-	+	+
Growth in anaerobic jar	-	+	-	-	-
Indole production					
Additional tests					

Table 15: Morphological and biochemical characteristics obtained by enrichment of isolates from colonised alder leaves with homogenised alder leaves in a mineral medium. Washings were cultured on selective agars.

(continued ...)

Isolate number / Characteristic	116	117	118	119	120
Medium of isolation	PSS ⟶ microaerophilic incubation ⟶				NA
Colony pigment and morphology			yellow		
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	+	+	+	+	+
Growth in anaerobic jar	-	-	-	-	-
Indole production					
Additional tests					

Table 15: Morphological and biochemical characteristics obtained by enrichment of isolates from colonised alder leaves with homogenised alder leaves in a mineral medium. Washings were cultured on selective agars.

(continued ...)

Isolate number / Characteristic	121	122	123	124	125	126
Medium of isolation	NA	NA	TA	NA	TA	OA
Colony pigment and morphology						purple rubbery
Gram stain	-	-	-	+	-	-
Morphology	rod	rod	rod	rod spores	rod	rod
Catalase	+	+	+	+	-	+
Oxidase	+	+	+	-	-	+
Motility	+	+	-	+	-	
Glucose fermentation	-	-	acid gas	-	-	-
Fluorescent pigment production	+	+	-	-	-	-
Growth in anaerobic jar	-	-	-	+		-
Indole production						
Additional tests						

Table 15: Morphological and biochemical characteristics obtained by enrichment of isolates from colonised alder leaves with homogenised alder leaves in a mineral medium. Washings were cultured on selective agars.

Isolate number / Characteristic	127	128	129	130	131
Medium of isolation	← cellophane disc → PSS and microaerophilic incubation				
Colony pigment and morphology					
Gram stain	-	-	+	-	-
Morphology	rod	rod	rod spores	rod	rod
Catalase	-	+	+	+	+
Oxidase	+	-	+	-	-
Motility	+	-	+	-	-
Glucose fermentation	-	acid gas	acid	acid gas	acid
Fluorescent pigment production	+	-	-	-	+
Growth in anaerobic jar	-	+	+	+	-
Indole production	-	-	-	-	-
Additional tests	API FAP	1 = + 2 = + FAP	FAP	1 = + 2 = + API FAP	

Table 16: Morphological and biochemical characteristics of organisms on decaying alder leaves enriched in mineral medium with cellophane discs or wicks. The suspensions of organisms subsequently removed from the cellophane were cultured on PSS with microaerophilic incubation

(continued ...)

Isolate number / Characteristic	132	133	134	135
Medium of isolation	← cellophane wick → PSS and microaerophilic incubation			
Colony pigment and morphology				
Gram stain	-	-	-	-
Morphology	rod	rod	rod	rod
Catalase	+	+	+	+
Oxidase	+	+	-	+
Motility	+	+	+	+
Glucose fermentation	-	acid	-	-
Fluorescent pigment production	-	-	-	+
Growth in anaerobic jar	-	-	-	-
Indole production	-	-		-
Additional tests	API	API FAP	FAP	API FAP

Table 16: Morphological and biochemical characteristics of organisms on decaying alder leaves enriched in mineral medium with cellophane discs or wicks. The suspensions of organisms subsequently removed from the cellophane were cultured on PSS with microaerophilic incubation

(continued ...)

Isolate number / Characteristic	136	137	138*	139	140
Medium of isolation	← cellophane disc → PSS & microaerophilic incubation				
Colony pigment and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	-	+	+
Oxidase	+	+	-	+	+
Motility	+	+		+	+
Glucose fermentation	-	-		-	-
Fluorescent pigment production	-	+		-	-
Growth in anaerobic jar	-	-		-	-
Indole production	-	-		-	-
Additional tests	API	API		API	API FAP

Table 16: Morphological and biochemical characteristics of organisms on decaying alder leaves enriched in mineral medium with cellophane discs or wicks. The suspensions of organisms subsequently removed from the cellophane were cultured on PSS with microaerophilic incubation

(continued ...)

Isolate number / Characteristic	141	142	143
Medium of isolation	cellophane disc ⟶ ⟵ PSS ⟶		
Colony pigment and morphology			
Gram stain	-	-	-
Morphology	rod	rod	rod
Catalase	+	+	+
Oxidase	+	+	+
Motility	+	+	+
Glucose fermentation	-	-	-
Fluorescent pigment production	+	+	+
Growth in anaerobic jar	-	-	-
Indole production	-	-	-
Additional tests	API FAP	API FAP	API

Table 16: Morphological and biochemical characteristics of organisms on decaying alder leaves enriched in mineral medium with cellophane discs or wicks. The suspensions of organisms subsequently removed from the cellophane were cultured on PSS with microaerophilic incubation

Isolate number / Characteristic	144	145	146	147*
Medium of isolation	← leaf homogenate agar →			
Colony pigment and morphology		yellow spreading	yellow spreading	
Gram stain	+	-	-	-
Morphology	rod spores	rod	rod	rod
Catalase	+	+	+	+
Oxidase	-	-	-	-
Motility	+	+	+	+
Glucose fermentation	acid	acid	-	acid
Fluorescent pigment production	-	-	-	-
Growth in anaerobic jar	-	-	-	-
Indole production	-	-	-	-
Additional tests		3=polar flagella FAP 9 = - 7 = + 8 = 0 10a = + b = + c = + d = - e = -	3=polar flagella 9 = - 7 = +	

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

(continued ...)

Isolate number / Characteristic	148*	149	150*	151
Medium of isolation	← leaf homogenate agar →			
Colony pigment and morphology				yellow spreading
Gram stain		+		-
Morphology		rod spores		rod
Catalase		+		+
Oxidase		-		-
Motility		+		+
Glucose fermentation		acid		acid
Fluorescent pigment production		-		-
Growth in anaerobic jar		-		-
Indole production		-		-
Additional tests		FAP		3=polar flagella FAP 9 = - 7 = - 10a = + b = + c = + d = - e = -

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

(continued ...)

Isolate number / Characteristic	152	153	154	155
Medium of isolation	← leaf homogenate agar →			
Colony pigment and morphology	coral pink	coral pink	coral pink	coral pink
Gram stain	+	+	+	+
Morphology	rod branched	rod branched	rod branched	rod branched
Catalase	+	+	+	+
Oxidase	-	-	-	-
Motility	-	-	-	-
Glucose fermentation	acid	-	-	-
Fluorescent pigment production	-	-	-	-
Growth in anaerobic jar	-	-	-	-
Indole production	-	-	-	-
Additional tests	3=branched rod 4 = - 5 = - FAP	5 = - FAP	4 = - 5 = - FAP	4 = - 5 = - FAP

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

(continued ...)

Isolate number / Characteristic	156	157	158*	159
Medium of isolation	← leaf homogenate agar →			
Colony pigment and morphology	coral pink		yellow spreading	
Gram stain	+	-	-	-
Morphology	rod branched	rod	rod	rod
Catalase	+	+	+	+
Oxidase	-	-	+	+
Motility	-	+		+
Glucose fermentation	acid	acid	acid	acid
Fluorescent pigment production	-	-	-	+
Growth in anaerobic jar	-	+	-	-
Indole production	-	+	-	-
Additional tests	4 = - 5 = - FAP	FAP	9 = -	API

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

(continued ...)

Isolate number / Characteristic	160	161*	162	163
Medium of isolation	leaf homogenate agar		PM	leaf homogenate agar
Colony pigment and morphology	yellow			
Gram stain	-	+	-	-
Morphology	rod	rod	rod	rod
Catalase	+	+	+	+
Oxidase	+	+	+	+
Motility	-	+	+	+
Glucose fermentation	acid	acid	acid	acid
Fluorescent pigment production	-	-	+	+
Growth in anaerobic jar	+	+	-	+
Indole production	+	-	-	-
Additional tests	API		API	API FAP

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

(continued ...)

Isolate number / Characteristic	164	165*	166	167*
Medium of isolation	← leaf homogenate agar			
Colony pigment and morphology				
Gram stain	-		-	
Morphology	rod		rod	
Catalase	+		+	
Oxidase	+		+	
Motility	+		+	
Glucose fermentation	acid		acid	
Fluorescent pigment production	+		-	
Growth in anaerobic jar	+		-	
Indole production	-		-	
Additional tests	6 = - API		API FAP	

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

(continued ...)

Isolate number / Characteristic	168	169	170	171
Medium of isolation	← leaf homogenate agar			
Colony pigment and morphology				
Gram stain	-	-	-	-
Morphology	rod	rod	rod	rod
Catalase	+	+	+	+
Oxidase	-	-	+	+
Motility	+	-	+	+
Glucose fermentation	acid	acid	acid	acid
Fluorescent pigment production	-	-	+	-
Growth in anaerobic jar	-	-	-	-
Indole production	-	+	-	-
Additional tests	2 = + API	1 = + 2 = + 4 = - 5 = - FAP		

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

(continued ...)

Isolate number / Characteristic	172	173	174	175	176
Medium of isolation	← leaf homogenate agar →				
Colony pigment and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	acid	-	acid	acid	acid
Fluorescent pigment production	-	+	-	+	-
Growth in anaerobic jar	+	-	-	-	-
Indole production	+	-	-	-	-
Additional tests	3 = peritrichous flagella API FAP	API	API FAP	API	API

Table 17: Morphological and biochemical characteristics of isolates obtained by enrichment of colonised alder leaves in mineral medium containing sterile alder leaves. Washings obtained from removal of the enriched organisms from the leaf surfaces were plated onto leaf homogenate agar and paste medium

Isolate number / Characteristic	177	178	179	180	181
Medium of isolation	←		TSBA		→
Colony pigment and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	acid	acid	acid	acid	acid
Fluorescent pigment production	+	+	+	+	+
Growth in anaerobic jar	-	-	-	-	+
Indole production	-	-	+	+	+
Additional tests	API	API	API FAP	API	API

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Characteristic	182	183	184	185
Medium of isolation	← TSBA →			
Colony pigment and morphology				
Gram stain	-	-	-	-
Morphology	rod	rod	rod	rod
Catalase	+	+	+	+
Oxidase	+	+	+	+
Motility	+	+	+	+
Glucose fermentation	-	acid	-	acid
Fluorescent pigment production	-	+	-	+
Growth in anaerobic jar	-	-	-	-
Indole production	-	+	-	+
Additional tests	API	API	API FAP	API

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Characteristic	186	187	188	189	190
Medium of isolation	←		TSBA		→
Colony pigment yellow and morphology					
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	-	acid	acid	-	acid
Fluorescent pigment production	+	+	+	-	-
Growth in anaerobic jar	-	-	-	-	-
Indole production	-	-	-	-	-
Additional tests	3=polar flagella API	API	API	4 = - 5 = - FAP	API

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Characteristic	191	192*	193	194
Medium of isolation	←		TSBA	→
Colony pigment and morphology			yellow	
Gram stain	-		-	-
Morphology	rod		rod	rod
Catalase	+		+	+
Oxidase	+		+	+
Motility	+		+	+
Glucose fermentation	acid		-	-
Fluorescent pigment production	-		-	-
Growth in anaerobic jar	-		-	-
Indole production	-	-	-	-
Additional tests	API		3=single polar flagellum 7 = + API FAP 9 = - 10a = + b = + c = - d = - e = -	API

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Characteristic	195*	196	197	198	199
Medium of isolation	←		TSBA		→
Colony pigment and morphology					
Gram stain		-	-	-	-
Morphology		rod	rod	rod	rod
Catalase		+	+	+	+
Oxidase		+	+	+	+
Motility		+	+	+	+
Glucose fermentation		-	-	-	acid
Fluorescent pigment production		-	-	-	-
Growth in anaerobic jar		-	-	-	-
Indole production		-	-	-	-
Additional tests		API	API FAP	3=polar flagella	API

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Characteristic	200	201	202	203	204
Medium of isolation	←		TSBA		→
Colony pigment and morphology		growth firmly attached to medium. Hair-like			
Gram stain	-	+	-	-	-
Morphology	rod	rod spores	rod	rod	rod
Catalase	+	+	+	-	-
Oxidase	+	-	+	+	+
Motility	+	+	+	+	+
Glucose fermentation	+	acid	-	-	-
Fluorescent pigment production	-	-	-	-	-
Growth in anaerobic jar	-	+	-	-	+
Indole production	+	-	-	+	-
Additional tests	3=polar flagella API	FAP	API	API	3=polar flagella API

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Characteristic	205	206	207	208	209
Medium of isolation	← TSBA →				
Colony pigment and morphology			mucoid		
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	-	-	-
Motility	+	+	-	+	+
Glucose fermentation	-	-	acid	acid	acid gas
Fluorescent pigment production	-	+	-	-	-
Growth in anaerobic jar	-	-	+	-	+
Indole production	-	-	+	-	+
Additional tests	FAP	2 = - 4 = - 5 = - FAP	2 = + FAP	2 = + FAP	1 = + 4 = - 5 = - FAP

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Charac-teristic	210	211	212	213	214
Medium of isolation	← TSBA →				
Colony pigment and morphology					
Gram stain	-	-	-	+	-
Morphology	rod	rod	rod	rods spores	rod
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	+
Motility	+	+	+	-	+
Glucose fermentation	-	acid	acid	acid	acid
Fluorescent pigment production	-	-	+	-	-
Growth in anaerobic jar	-	-	-	-	-
Indole production	-	-	-	+	-
Additional tests	API FAP	4 = + FAP	4 = + FAP		FAP

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

(continued ...)

Isolate number / Characteristic	215	216	217	218	219
Medium of isolation	←		TSBA		→
Colony pigment and morphology					spreading growth
Gram stain	+	+	-	-	-
Morphology	rod spores	rod spores	rod	rod	rod
Catalase	+	+	+	+	-
Oxidase	+	-	-	-	-
Motility	+	+	+	+	-
Glucose fermentation	acid	acid	acid	-	acid
Fluorescent pigment production	-	-	-	-	-
Growth in anaerobic jar	-	-	-	-	+
Indole production	-	-	-	-	-
Additional tests	4 = +	4 = + 5 = - 7 = -	4 = + 5 = - 7 = - FAP	4 = + 5 = - 7 = - FAP	FAP

Table 18: Morphological and biochemical characteristics of isolates obtained by imprinting of colonised leaves onto TSB agar and either incubating media with leaves for the full incubation period, or removal of leaves after 24 hours and further incubation of the imprinted agar.

Isolate number / Characteristic	220	221	222	223	224
Medium of isolation	← damp filter paper →				
Colony pigment and morphology	← yellow spreading →				
Gram stain	-	-	-	-	-
Morphology	rod	rod	rod	rod	rod
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Motility	-	-	-	-	-
Glucose fermentation	-	-	-	-	-
Fluorescent pigment production	-	-	-	-	-
Growth in anaerobic jar					
Indole production					
Additional tests	FAP	FAP	FAP	FAP	FAP

Table 19: Morphological and biochemical characteristics of isolates obtained by enrichment in mineral medium containing dog fur, of a suspension of isolates obtained from colonised alder leaves which had been dried and rehydrated in water. Washings and dog fur were plated onto chitin agar, oatmeal agar and damp filter paper after a 2-6 week enrichment.

(continued ...)

Isolate number / Characteristic	225	226
Medium of isolation	chitin agar →	
Colony pigment and morphology	grey mycelium	white mycelium
Gram stain		
Morphology		
Catalase		
Oxidase		
Motility		
Glucose fermentation		
Fluorescent pigment production		
Growth in anaerobic jar		
Indole production		
Additional tests		

Table 19: Morphological and biochemical characteristics of isolates obtained by enrichment in mineral medium containing dog fur, of a suspension of isolates obtained from colonised alder leaves which had been dried and rehydrated in water. Washings and dog fur were plated onto chitin agar, oatmeal agar and damp filter paper after a 2-6 week enrichment.

Using a combination of techniques 226 organisms were isolated. All isolates were tested using a standard set of morphological and biochemical tests and further characterisation was assisted by the use of API20NE strips and fatty acid profiling (tables 14-20). Negatively stained TEM preparations were used to assess flagellar number and insertion where necessary. The flagellar arrangement of isolate number 151 is shown in figure 24.

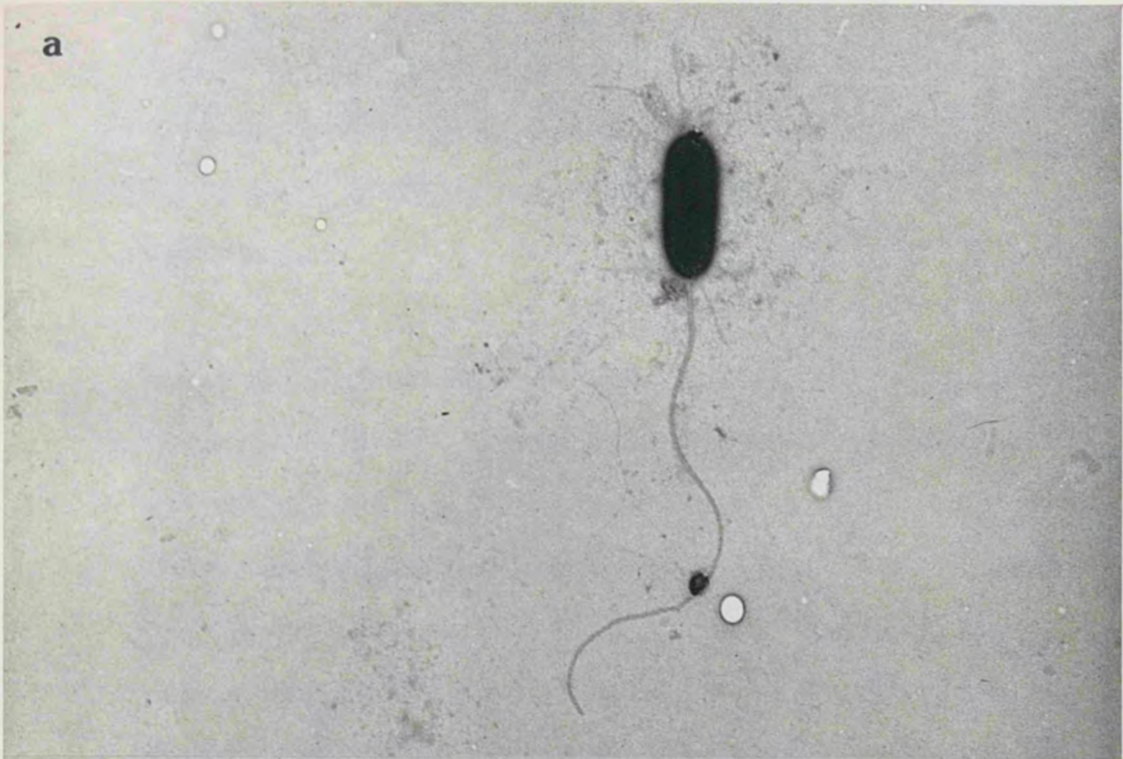
Streptomycete-like organims are often difficult to characterise using conventional methods and the greater emphasis is placed on accurate morphological description (Shirling and Gottlieb 1966; Willoughby, personal communication). The characteristics of isolates 225 and 226 are shown in table 20.

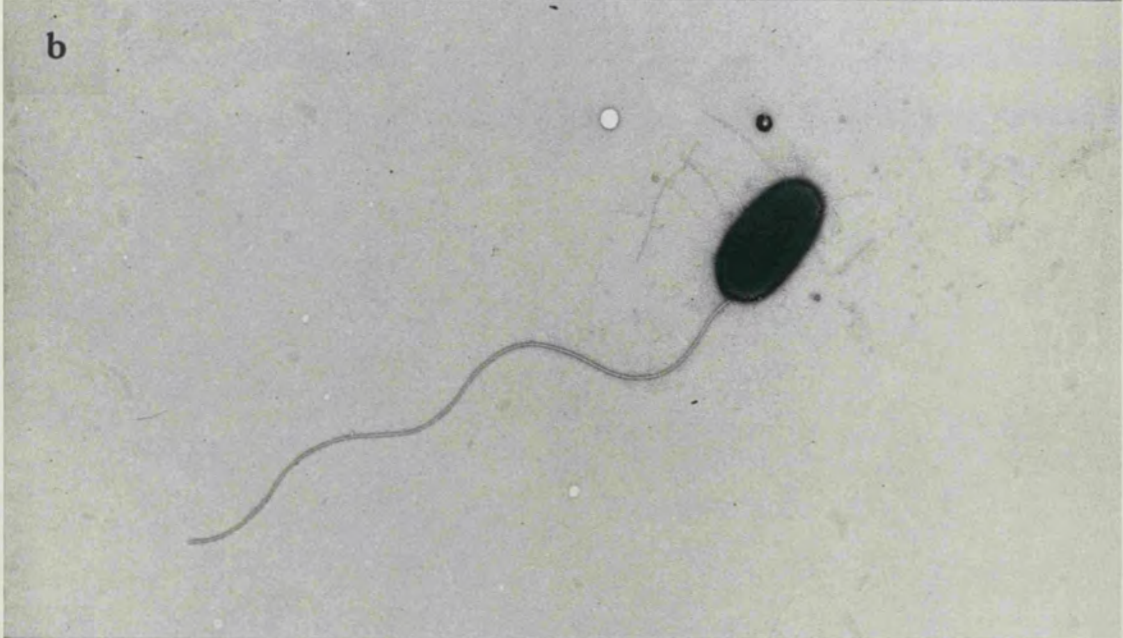
	Isolate Number	
Characteristic	225	226
Colony surface colour	grey	white
Colony base colour on yeme	yellow	yellow
Spore chain morphology	spiral	straight
Spore ornamentation SEM		smooth

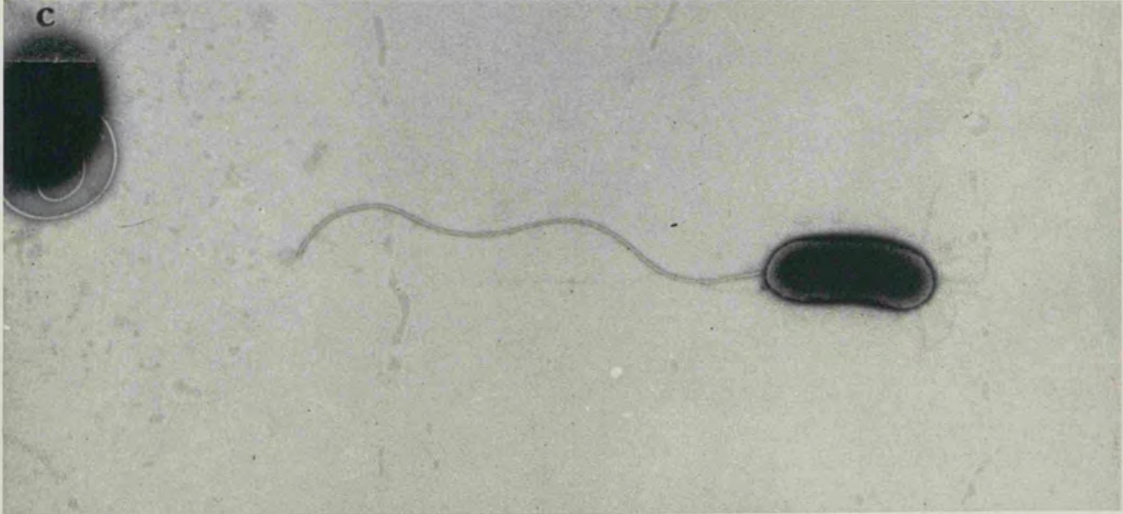
Table 20: Morphological characteristics of streptomycete-like isolates.

On the evidence obtained from the basic morphological and biochemical testing organisms were characterised to family level wherever possible. To further assist in

Figure 24: Negatively stained TEM preparation of Isolate Number 151 to show flagellar arrangement and insertion. Note presence of fimbriae. Magnification 7000x.


a


b


c

characterisation, Gram negative oxidase positive organisms were also inoculated onto API 20NE strips. Fatty acid profiles were also obtained for some isolates.

Fatty acid profiling served 3 purposes; as a means of comparison with results obtained using standard morphological and biochemical tests, to characterise those isolates displaying characteristics inappropriate for characterisation of API 20NE strips and to characterise and group those isolates for which no determination was made. 85% of isolates were thus characterised, 11% died before testing was complete and no determination was made for the remaining 4% (table 21).

Isolate number	Identity (family, genus or genus & species)
1	Pseudomonas
2	"
3	"
4	"
5	Unknown
6*	
7	Pseudomonas
8	"
9	"
10	"
11	Pseudomonas
12*	
13*	
14	Pseudomonas
15	Janthinobacterium
16*	
17*	
18	Pseudomonas
19	"
20	"
21	"
22	"
23	"
24*	
25	Pseudomonas
26	"
27	"

Isolate number	Identity (family, genus or genus & species)
28	Micrococcaceae
29*	
30	Pseudomonas
31	Pseudomonas fluorescens
32	Pseudomonas
33	"
34	Pseudomans fluorescens
35	Pseudomonas
36	"
37	Pseudomonas fluorescens
38	" "
39	" "
40	Pseudomonas
41	"
42	"
43	"
44	Pseudomonas fluorescens
45	" "
46	Pseudomonas
47	Pseudomonas fluorescens
48	" "
49	" "
50	Unknown
51*	
52	Pseudomonas
53	Pseudomonas fluorescens
54	Pseudomonas

Isolate number	Identity (family, genus or genus & species)
55	Pseudomonas fluorescens
56	Pseudomonas
57	"
58	"
59	Pseudomonas fluorescens
60*	
61	Pseudomonas fluorescens
62	" "
63	Pseudomonas
64	"
65	Pseudomonas fluorescens
66	Pseudomonas
67	Unknown
68*	
69	Pseudomonas
70	Pseudomonas
71	Pseudomonas fluorescens
72	Pseudomonas
73	Pseudomonas fluorescens
74	Pseudomonas
75	"
76*	
77	Pseudomonas
78*	
79	Pseudomonas
80	"

Isolate number	Identity (family, genus or genus & species)
81	Pseudomonas fluorescens
82	Pseudomonas
83	"
84	"
85	"
86	"
87*	
88	Pseudomonas
89	Pseudomonas
90	"
91	"
92	"
93	"
94	"
95	"
96	"
97	"
98	"
99*	
100	Pseudomonas
101	"
102	"
103	"
104	Unknown
105	Pseudomonas
106	"
107	"

Isolate number	Identity (family, genus or genus & species)
108*	
109	Pseudomonas
110	"
111	"
112	"
113	"
114	"
115	"
116	"
117	Micrococcaceae
118	Pseudomonas
119	Pseudomonas
120	"
121	"
122	"
123	Unknown
124	Bacillus
125*	
126	Janthinobacterium
127	Pseudomonas testosteroni
128	Proteus vulgaris
129	Bacillus
130	Aeromonas hydrophila
131	Unknown
132	Pseudomonas
133	"
134	Xanthomonas maltophilia

Isolate number	Identity (family, genus or genus & species)
135	Pseudomonas
136	"
137	"
138*	
139	Pseudomonas
140	"
141	"
142	"
143	"
144	Bacillus
145	Cytophaga
146	Unknown
147*	
148*	
149	Bacillus
150*	
151	Xanthomonas
152	Rhodococcus
153	"
154	"
155	"
156	"
157	Erwinia
158	Unknown
159	Pseudomonas
160	Xanthomonas
161	Unknown

Isolate number	Identity (family, genus or genus & species)
162	Pseudomonas fluorescens
163	" "
164	Pseudomonas putida
165*	
166	Pseudomonas putida
167*	
168	Alcaligenes
169	Erwinia
170	Pseudomonas
171	"
172	Alcaligenes
173	Pseudomonas
174	Aeromonas hydrophila
175	Pseudomonas
176	"
177	"
178	Pseudomonas
179	"
180	"
181	"
182	"
183	"
184	Xanthomonas maltophilia
185	Pseudomonas
186	"
187	"
188	Pseudomonas cepacia

Isolate number	Identity (family, genus or genus & species)
189	Pseudomonas
190	Pseudomonas putida
191	Pseudomonas
192*	
193	Xanthomonas
194	Pseudomonas testo/alcalis
195*	
196	Pseudomonas
197	Pseudomonas testosteroni
198	Pseudomonas cepacia
199	Pseudomonas testo/alcalis
200	" "
201	Bacillus
202	Pseudomonas testo/alcalis
203	" "
204	" "
205	" "
206	" "
207	Klebsiella
208	Escherichia coli
209	Klebsiella
210	Xanthomonas maltophilia
211	Pseudomonas
212	"
213	Bacillus
214	Pseudomonas
215	Bacillus

Isolate number	Identity (family, genus or genus & species)
216	"
217	Pseudomonas
218	"
219	Proteus
220	Cytophaga
221	
222	"
223	"
224	"
225	Streptomyces (grey)
226	Streptomyces (white)

Table 21: Identifications of isolates.

* isoloates which died before characterisation was complete

When identifying large numbers of microorganisms as in an ecological study the time spent identifying, the availability and cost of equipment and media, and accuracy are important considerations.

The biochemical and morphological considerations selected for initial characterisation of alder leaf surface bacterial isolates are routinely used for this purpose and the equipment and media was therefore readily available. The factor limiting use of a broader range of biochemical tests is time. The cost of processing large numbers of isolates is difficult to quantify since there is usually no large capital outlay required and routinely available media are used. Generally the cost of media is far outweighed by the time taken to prepare the media, perform the tests and then to dispose of, or wash contaminated equipment.

Selection of further appropriate biochemical testing of isolates is dependant on results obtained and their interpretation. The use of API 20NE strips to assist in identification of Gram negative, oxidase positive organisms helped reduce the number of isolates which required further biochemical testing in order to facilitate characterisation. Additionally, the strips required no specialised equipment and results were obtained within 24 to 48 hours.

Fatty acid profiling requires relatively little

expertise in bacterial taxonomy or retention of determinative tests and the interpretation of results obtained from them. Stead (1987, in press) demonstrated that at specific level of more than 400 reference strains of plant pathogenic bacteria from the National Collection of Plant Pathogenic Bacteria (NCPPB) UK characterisation was appropximately 98% successful. It is, however, less simple to evaluate the accuracy of characterisation of fatty acid profiling of organisms isolated in an ecological study since these organisms are frequently not identified and are also not as well represented in recognised culture collections as are the economically important and clinically important microorganisms.

The low similarity indices for fatty acid profiling of some isolates (figs. 25-39) indicates that the characterisation of these isolates is not wholly accurate, but that it is the closest fit from within the same group of bacteria. Generally, values above 0,5 are accurate and above 0,1 accurate only to genus level. Reference to standard texts such as Bergey's Manual of Systematic Bacteriology enables selection of a few key tests by which identity can be more precisely determined. Characterisation by either API 20NE strips or fatty acid profiling can only be as good as the library developed.

Qualitative and quantitative composition of bacterial

fatty acids provides a stable phenotypic character and even when specific determination cannot be made the profile can be used to classify the organism to some extent (Sasser and Smith, 1987; Moss, 1981; Moss et al. 1980). This type of information is not available using API 2ONE strips. Results from API 2ONE strips may be further complicated by inconclusive results or problems with contamination.

Fatty acid profiling was therefore found to be a rapid, accurate and versatile method for determination of large numbers of bacteria. Standard morphological and biochemical testing and use of API 2ONE strips although more readily available and less expensive, are useful only for a restricted range of organisms.

Streptomycetes and Actinomycetes commonly occur in leaves and detritus (Makkar and Cross, 1982; Willoughby, 1969) and their presence on degrading alder leaves was therefore sought by drying and enriching of previously colonised leaves in mineral medium with small quantities of dog fur. Dog fur is a source rich in keratin and would therefore select for keratinophilic Streptomyces spp. Washings and strands of dog fur from the enriched culture were plated onto sterile damp filter paper and onto chitin agar. Growth of Streptomyces spp occurred within 2 to 3 weeks of incubation at room temperature and duplicate cultures were obtained by replica plating chitin agar or filter

paper cultures onto fresh damp filter paper which was incubated in petri dishes sealed in order to maintain high humidity.

Isolation of Streptomyces spp was only accomplished using the above method although washings from other enrichments which used cellophane or leaf homogenate were also plated out onto media suitable to promote growth of Streptomyces sp. Enrichment with dog fur or drying of leaves prior to enrichment, or both, therefore accounted for the successful isolation of these organisms.

Cytophaga was isolated by enriching with dog fur and subcultured in the same manner described for Streptomyces spp. Growth of Cytophaga was difficult to visualise on chitin agar, but areas where growth had occurred on filter paper appeared distinctly yellow. Agar slants and broth cultures were loop inoculated from growth on filter paper and the isolates were stored as described in Methods and Materials.

Cytophaga has been isolated in enrichments with cellophane and leaf homogenate, but they appeared infrequently and growth was sparse. This indicates that other organisms were preferentially enriched and that growth of Cytophaga was inhibited by the more rapid growth and possible antagonism of other organisms. Enrichment with dog fur was therefore

satisfactory as only two groups of microorganisms were obtained and neither appeared to significantly inhibit growth of the other.

Further investigation of nutritional versatility, enzyme production and physical tolerances of isolates from a particular habitat is often desirable so that the ecology of the environment can be better understood and the behaviour and relationships between the constituent flora predicted. Representative members of the flora must first be isolated and characterised before any tests can be conducted. The question of how representative a set of isolates is cannot be answered simply. The approach adopted in this investigation was that bacteria are present in almost all habitats, often saprophytically, and that many of the isolates would therefore be of no significance in leaf breakdown. However, by enrichment of microorganisms using pure substrates, or low concentration of leaf homogenate it would be likely that a more representative set of isolates would be obtained. The work therefore set out to obtain as many isolates as was practical using a variety of techniques. Numbers of isolates of any one particular genus were therefore considered irrelevant and any genus isolated more than once (to avoid use of possible contaminants) was considered to have been present on the degrading alder leaves. Streptomyces spp, although only listed once each in tables 19 and 21 occurred more than once on agar plates but replicates

were not listed as additional isolates as morphological examination showed only the presence of two different species.

A representative of each genus isolated from alder leaves was selected for further testing (table 22) and fatty acid profiles of these organisms are shown in figs. 25-39.

Isolate number	Identity
193	Xanthomonas
179	Ps. cepacia/chloraphis
174	Aeromonas
210	Ps. maltophilia
168	Alcaligenes
197	Ps. testosteroni
166	Ps. putida
169	Erwinia
201	Bacillus (spreading growth)
149	Bacillus (discrete colonies)
155	Rhodococcus
163	Ps. fluorescens
222	Cytophaga
219	Proteus
15	Janthinobacterium
225	Streptomyces (grey)
226	Streptomyces (white)

Table 22: Bacterial isolates selected for further work

Figure 25: Integrated fatty acid profile of Isolate number 197

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350G12870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 25
ID: 1415
Name: UN-INGRID 21C **Isolate 197**

Date of report: 01-APR-87 06:06:19
Date of run: 01-APR-87 06:06:19

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.589	30604000	0.061	. . .	7.075	SOLVENT PEAK	. . .	< min rt	
2.396	512	0.029	. . .	8.825	. . . .	. . .	< min rt	
2.936	501	0.028	1.175	10.000	10:0	0.22	ECL deviates 0.000	Reference -0.009
3.938	7514	0.030	1.085	11.429	10:0 3OH	3.10	ECL deviates 0.006	
4.426	5895	0.031	1.057	12.000	12:0	2.37	ECL deviates 0.000	Reference -0.002
6.808	619	0.037	0.985	13.999	14:0	0.23	ECL deviates -0.001	Reference -0.002
8.292	2584	0.040	0.960	15.000	15:0	0.94	ECL deviates 0.000	Reference -0.001
9.618	89223	0.042	0.944	15.819	16:1 CIS 9	32.06	ECL deviates 0.002	
9.911	77247	0.043	0.941	16.000	16:0	27.67	ECL deviates 0.000	Reference -0.002
10.284	896	0.044	0.937	16.220	15:0 2OH	0.32	ECL deviates 0.003	
11.418	14741	0.046	0.928	16.890	17:0 CYCLO	5.21	ECL deviates 0.002	Reference -0.000
11.606	4214	0.044	0.926	17.001	17:0	1.49	ECL deviates 0.001	Reference -0.001
11.696	5779	0.047	0.926	17.053	16:1 2OH	2.04	ECL deviates 0.006	
12.016	8090	0.047	0.924	17.237	16:0 2OH	2.84	ECL deviates 0.002	
13.033	58196	0.046	0.918	17.823	18:1 CIS 11/t 9/t 6	20.34	ECL deviates 0.001	Sum In Feature 7
13.336	1327	0.047	0.517	17.998	18:0	0.46	ECL deviates -0.002	Reference -0.004
14.902	2018	0.049	0.913	18.902	19:0 CYCLO C11-12 .	0.70	ECL deviates 0.002	Reference 0.001
18.020	26351	0.049	. . .	20.715	. . . .	. . .	> max rt	
19.636	12662	0.743	. . .	21.653	. . . .	. . .	> max rt	
*******	58196	. . .	. . .	. . .	SUMMED FEATURE 7 . .	20.34		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30604000	278844	278844	100.00	262710	9	0.004	0.003

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES	SIMILARITY
Pseudomonas	0.826
P. testosteroni	0.826
P. corrugata	0.010
P. syringae	0.009
P. s. tabaci	0.008
P. s. tagetes	0.007
P. s. phaseolicola	0.007
Chromobacterium	0.004
C. violaceum	0.004

WED 01-APR-87 06:00:26

BOTTLE: 25 ID#: 1415
SAMPLE TYPE: SAMPLE
UN-INGRID 21C
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

Figure 26: Integrated fatty acid profile of Isolate number 179.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87121591
Bottle: 9
ID: 1180
Name: UN INGRID 3C (PSEUD CEPACIA) **Isolate 179**

Date of report: 21-JAN-87 19:21:54
Date of run: 21-JAN-87 19:21:54

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.613	38789000	0.077	. . .	7.046	SOLVENT PEAK	. . .	< min rt	
1.830	6314	0.028	. . .	7.505	. . . .	. . .	< min rt	
1.958	454	0.024	. . .	7.777	. . . .	. . .	< min rt	
2.143	1529	0.026	. . .	8.169	. . . .	. . .	< min rt	
2.223	989	0.025	. . .	8.339	. . . .	. . .	< min rt	
2.367	1489	0.026	. . .	8.645	. . . .	. . .	< min rt	
2.453	678	0.034	. . .	8.828	. . . .	. . .	< min rt	
2.515	1153	0.029	. . .	8.960	. . . .	. . .	< min rt	
2.647	657	0.032	. . .	9.240	. . . .	. . .		
3.004	7612	0.031	1.149	10.000	10:0	0.80	ECL deviates 0.000	Reference 0.000
3.317	10013	0.029	. . .	10.480	. . . .	. . .		
3.833	4072	0.033	. . .	11.203	. . . .	. . .		
4.033	55733	0.031	1.072	11.433	10:0 3OH	5.47	ECL deviates 0.010	
4.528	21751	0.050	1.046	12.000	12:0	2.08	ECL deviates 0.000	Reference 0.005
4.922	8726	0.033	. . .	12.359	. . . .	. . .		
5.064	8513	0.034	1.028	12.488	unknown 12.486 . . .	0.80	ECL deviates 0.002	
5.115	2283	0.031	. . .	12.535	. . . .	. . .		
5.237	1679	0.034	. . .	12.646	. . . .	. . .		
5.869	55935	0.039	1.004	13.183	12:0 2OH	5.14	ECL deviates 0.005	
6.019	21405	0.037	1.001	13.297	12:1 3OH	1.96	ECL deviates 0.008	
6.237	68528	0.037	0.996	13.462	12:0 3OH	6.24	ECL deviates 0.007	
6.698	4154	0.047	0.986	13.812	13:0 ISO 2OH	0.37	ECL deviates -0.002	
6.868	2353	0.061	. . .	13.941	. . . .	. . .		
6.946	3428	0.039	0.981	13.999	14:0	0.31	ECL deviates -0.001	Reference 0.004
7.356	3626	0.039	. . .	14.273	. . . .	. . .		
7.701	2828	0.044	0.970	14.503	unknown 14.503 . . .	0.25	ECL deviates 0.000	
8.441	2347	0.044	0.960	14.998	15:0	0.21	ECL deviates -0.002	Reference 0.002
8.739	1114	0.041	. . .	15.180	. . . .	. . .		
8.906	12884	0.043	. . .	15.283	. . . .	. . .		
9.251	11062	0.044	0.952	15.495	14:0 3OH/16:1 ISO I	0.96	ECL deviates 0.005	Sum In Feature 3
9.785	306430	0.047	0.947	15.823	16:1 CIS 9	26.56	ECL deviates 0.006	
10.079	280760	0.043	0.945	16.003	16:0	24.28	ECL deviates 0.003	Reference 0.006
11.143	1370	0.046	0.939	16.628	17:0 ISO	0.12	ECL deviates -0.001	Reference 0.003
11.591	75010	0.046	0.936	16.891	17:0 CYCLO	6.43'	ECL deviates 0.003	Reference 0.007
11.774	1307	0.050	0.936	16.999	17:0	0.11	ECL deviates -0.001	Reference 0.002
12.344	1685	0.050	. . .	17.326	. . . .	. . .		
12.691	1443	0.044	0.933	17.526	16:0 3OH	0.12	ECL deviates 0.006	
13.094	908	0.049	. . .	17.757	. . . .	. . .		
13.213	199320	0.047	0.931	17.825	18:1 TRANS 9/t6/c11	16.99	ECL deviates 0.000	Sum In Feature 7
13.511	7183	0.047	0.931	17.997	18:0	0.61	ECL deviates -0.003	Reference 0.000
13.656	2747	0.050	. . .	18.080	. . . .	. . .		
14.305	808	0.056	. . .	18.455	. . . .	. . .		
15.082	1061	0.048	0.930	18.903	19:0 CYCLO C11-12 .	0.09	ECL deviates 0.003	Reference 0.008
15.896	1101	0.061	0.931	19.376	unknown 19.368 . . .	0.09	ECL deviates 0.008	
16.053	1000	0.050	. . .	19.467	. . . .	. . .		
*******	11062	. . .	. . .	. . .	SUMMED FEATURE 3 . .	0.96		
*******	199320	. . .	. . .	. . .	SUMMED FEATURE 7 . .	16.99		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
38789000	1192836	1138281	95.43	1092898	10	0.004	0.004

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES SIMILARITY

Pseudomonas 0.481
P. chlororaphis . . . . 0.475 (P. fluorescens D)
P. corrugata 0.378
P. tolaasii 0.168

WED 21-JAN-87 19:19:48

BOTTLE: 9 ID#: 1180
SAMPLE TYPE: SAMPLE
UN INGRID 3C (PSEUD CEPACIA)
FILE NAME: DATA:F87121591

WORKFILE ID: A
WORKFILE NAME:

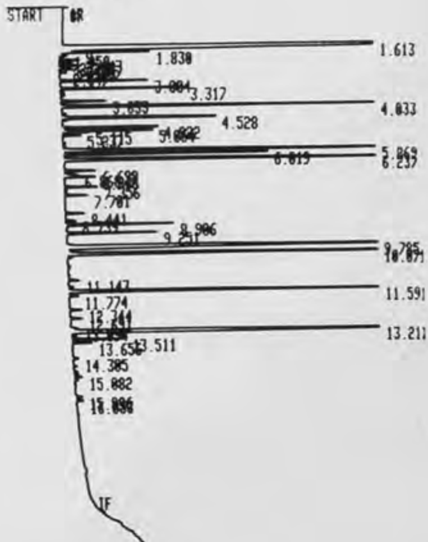


Figure 27: Integrated fatty acid profile of Isolate number 163.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 9
ID: 1399
Name: UN-INGRID 200 Isolate 163

Date of report: 31-MAR-87 21:27:59
Date of run: 31-MAR-87 21:27:59

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	29752000	0.059	. . .	7.061	SOLVENT PEAK	. . .	< min rt	
2.395	375	0.024	. . .	8.813	. . . .	. . .	< min rt	
2.454	435	0.027	. . .	8.941	. . . .	. . .	< min rt	
3.938	7760	0.030	1.081	11.427	10:0 3OH	2.88	ECL deviates 0.004	
4.426	7349	0.035	1.053	12.000	12:0	2.65	ECL deviates 0.000	Reference -0.005
5.742	14244	0.036	1.008	13.181	12:0 2OH	4.92	ECL deviates 0.003	
6.105	12311	0.036	0.999	13.459	12:0 3OH	4.22	ECL deviates 0.004	
6.809	1021	0.037	0.983	14.000	14:0	0.34	ECL deviates 0.000	Reference -0.003
8.291	919	0.043	0.958	14.999	15:0	0.30	ECL deviates -0.001	Reference -0.004
9.616	109400	0.042	0.942	15.817	16:1 CIS 9	35.35	ECL deviates 0.000	
9.911	86336	0.042	0.939	15.999	16:0	27.81	ECL deviates -0.001	Reference -0.003
11.419	9316	0.045	0.927	16.889	17:0 CYCLO	2.96	ECL deviates 0.001	Reference -0.001
11.605	1269	0.045	0.925	16.999	17:0	0.40	ECL deviates -0.001	Reference -0.004
13.034	55770	0.046	0.918	17.824	18:1 TRANS 9/t6/c11	17.56	ECL deviates -0.001	Sum In Feature 7
13.336	1916	0.046	0.917	17.999	18:0	0.60	ECL deviates -0.001	Reference -0.004
18.017	23320	0.046	. . .	20.719	. . . .	. . .	> max rt	
19.489	8535	0.535	. . .	21.578	. . . .	. . .	> max rt	
*******	55770	. . .	. . .	. . .	SUMMED FEATURE 7 . .	17.56		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
29752000	307611	307611	100.00	291638	7	0.004	0.002

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES — SIMILARITY

Pseudomonas 0.843
- P. chlororaphis . . . . 0.831 (P. fluorescens D)
- P. tolaasii 0.614
- P. fluorescens 0.576
  - P. f. A 0.545
  - P. f. B 0.505
  - P. f. G 0.411

Chromobacterium 0.238
- C. violaceum 0.238

Plesiomonas 0.010
- P. shigelloides 0.010

TUE 31-MAR-87 21:25:46

BOTTLE: 9 ID#: 1399
SAMPLE TYPE: SAMPLE
UN-INGRID 20D
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

START

1.593
3.938
4.426
5.742
6.105
6.809
8.291
9.616
9.911
11.605 11.419
13.034
13.336
18.017
19.489

Figure 28: Integrated fatty acid profile of Isolate number 219.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87121591 — Date of report: 22-JAN-87 01:37:07
Bottle: 21 — Date of run: 22-JAN-87 01:37:07
ID: 1192
Name: UN INGRID 1E (PROTEUS)

Isolate 219

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.613	39624000	0.079	. . .	7.033	SOLVENT PEAK	. . .	< min rt	
1.834	6243	0.028	. . .	7.501	. . . .	. . .	< min rt	
2.299	1963	0.030	. . .	8.487	. . . .	. . .	< min rt	
2.461	1301	0.030	. . .	8.830	. . . .	. . .	< min rt	
3.609	1542	0.029	1.116	10.919	unknown 10.928 . . .	0.22	ECL deviates -0.009	
4.533	4577	0.033	1.067	12.000	12:0	0.63	ECL deviates 0.000	Reference -0.001
6.704	1824	0.051	1.001	13.814	13:0 ISO 2OH	0.24	ECL deviates -0.000	
6.948	103480	0.041	0.995	13.999	14:0	13.27	ECL deviates -0.001	Reference -0.002
7.701	5135	0.043	0.981	14.502	unknown 14.503 . . .	0.65	ECL deviates -0.001	
8.445	8723	0.042	0.967	14.999	15:0	1.09	ECL deviates -0.001	Reference -0.002
8.739	2625	0.042	. . .	15.180	. . . .	. . .		
9.253	59513	0.043	0.955	15.495	14:0 3OH/16:1 ISO I	7.33	ECL deviates 0.005	Sum In Feature 3
9.709	1747	0.032	0.949	15.775	16:1 B	0.21	ECL deviates 0.001	
9.782	153450	0.043	0.948	15.819	16:1 CIS 9	18.75	ECL deviates 0.002	
9.929	2231	0.044	0.946	15.910	16:1 C	0.27	ECL deviates 0.002	
10.078	253420	0.042	0.944	16.001	16:0	30.83	ECL deviates 0.001	Reference -0.001
10.234	1297	0.066	. . .	16.093	. . . .	. . .		
11.426	1032	0.050	0.930	16.794	17:1 B	0.12	ECL deviates 0.002	
11.591	81467	0.045	0.928	16.891	17:0 CYCLO	9.74	ECL deviates 0.003	Reference 0.001
11.774	912	0.042	0.926	16.999	17:0	0.11	ECL deviates -0.001	Reference -0.004
13.094	1276	0.050	. . .	17.757	. . . .	. . .		
13.211	132280	0.046	0.915	17.824	18:1 TRANS 9/t6/c11	15.60	ECL deviates -0.001	Sum In Feature 7
13.512	2734	0.045	0.913	17.997	18:0	0.32	ECL deviates -0.003	Reference -0.005
14.301	1360	0.062	. . .	18.453	. . . .	. . .		
15.081	5324	0.049	0.907	18.903	19:0 CYCLO C11-12 .	0.62	ECL deviates 0.003	Reference 0.001
*******	59513	. . .	. . .	. . .	SUMMED FEATURE 3 . .	7.33		
*******	132280	. . .	. . .	. . .	SUMMED FEATURE 7 . .	15.60		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
39624000	825949	819391	99.21	776076	8	0.003	0.003

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES	SIMILARITY	
Proteus	0.718	
P. vulgaris	0.718	
P. mirabilis	0.296	
Klebsiella	0.495	
K. pneumoniae	0.495	
K. p. pneumoniae	0.495	
K. p. ozaenae	0.027	
Erwinia	0.322	
E. chrysanthemi	0.322	
E. c. IV	0.322	
E. c. VI	0.092	
E. c. III	0.036	
E. herbicola	0.059	(Enterobacter agglomerans)
E. h. herbicola	0.059	
E. salicis	0.010	

THU 22-JAN-87 01:34:41

BOTTLE: 21 ID#: 1192
SAMPLE TYPE: SAMPLE
UN INGRID 1E (PROTEUS)
FILE NAME: DATA:F87121591

WORKFILE ID: A
WORKFILE NAME:

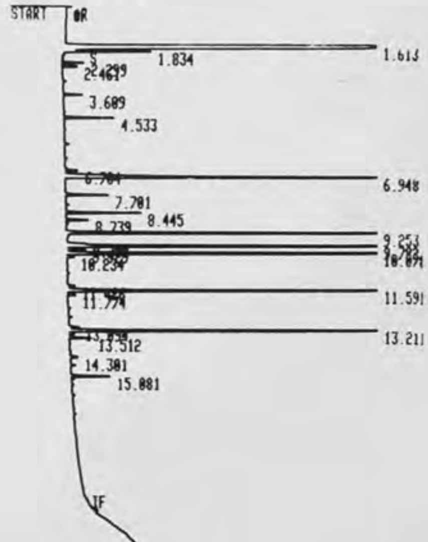


Figure 29: Integrated fatty acid profile of Isolate number 166.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 26
ID: 1416
Name: UN-INGRID23b0 Isolate 166

Date of report: 01-APR-87 06:35:23
Date of run: 01-APR-87 06:35:23

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.589	30295000	0.060	. . .	7.063	SOLVENT PEAK	. . .	< min rt	
2.395	396	0.025	. . .	8.820	. . . .	. . .	< min rt	
2.933	601	0.031	1.175	9.993	10:0	0.29	ECL deviates -0.007	
3.237	449	0.028	. . .	10.474	. . . .	. . .		
3.938	8150	0.030	1.085	11.430	10:0 3OH	3.69	ECL deviates 0.007	
4.425	4773	0.035	1.057	12.000	12:0	2.11	ECL deviates 0.000	Reference -0.004
5.741	11977	0.036	1.011	13.180	12:0 2OH	5.05	ECL deviates 0.002	
6.105	9919	0.037	1.001	13.459	12:0 3OH	4.15	ECL deviates 0.004	
6.809	792	0.038	0.985	14.000	14:0	0.33	ECL deviates -0.000	Reference -0.002
9.616	76845	0.044	0.944	15.817	16:1 CIS 9	30.28	ECL deviates -0.000	
9.675	12930	0.037	0.943	15.853	16:1 TRANS 9/15i2OH	5.09	ECL deviates -0.003	Sum In Feature 4
9.911	68266	0.043	0.941	15.999	16:0	26.81	ECL deviates -0.001	Reference -0.002
11.418	6037	0.045	0.928	16.889	17:0 CYCLO	2.34	ECL deviates 0.001	Reference -0.000
13.032	50344	0.048	0.918	17.823	18:1 CIS 11/t 9/t 6	19.29	ECL deviates 0.001	Sum In Feature 7
13.337	1504	0.046	0.917	17.999	18:0	0.58	ECL deviates -0.001	Reference -0.003
17.762	509	0.193	. . .	20.575	. . . .	. . .	> max rt	
18.020	25882	0.051	. . .	20.726	. . . .	. . .	> max rt	
*******	12930	. . .	. . .	. . .	SUMMED FEATURE 4 . .	5.09		
*******	50344	. . .	. . .	. . .	SUMMED FEATURE 7 . .	19.29		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30295000	252587	252138	99.82	239587	5	0.002	0.003

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES SIMILARITY

Pseudomonas 0.521
  P. putida 0.521
    P. p. biovar A 0.521
  P. chlororaphis . . . . 0.002 (P. fluorescens D)
  P. corrugata 0.002

WED 01-APR-87 06:32:37

BOTTLE: 26 ID#: 1416
SAMPLE TYPE: SAMPLE
UN-INGRID23BD
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

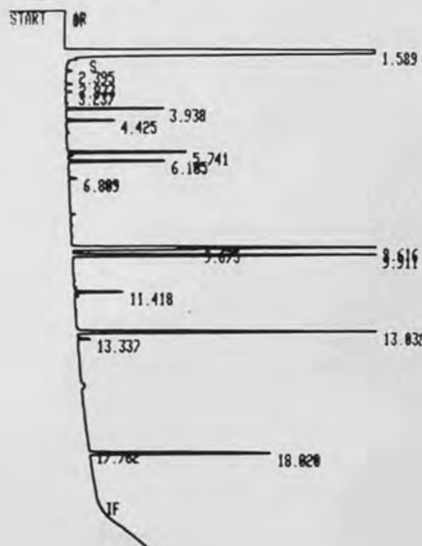


Figure 30: Integrated fatty acid profile of Isolate number 168.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 13
ID: 1403
Name: UN-INGRID 25aD **Isolate 168**

Date of report: 31-MAR-87 23:22:34
Date of run: 31-MAR-87 23:22:34

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	30230000	0.060	...	7.054	SOLVENT PEAK	...	< min rt	
2.398	535	0.026	...	8.815	....	...	< min rt	
3.523	609	0.028	...	10.917	....	...		
4.426	685	0.032	1.053	12.000	12:0	0.49	ECL deviates -0.000	Reference -0.005
5.742	3689	0.036	1.008	13.181	12:0 2OH	2.53	ECL deviates 0.003	
6.808	3963	0.044	0.983	14.000	14:0	2.65	ECL deviates 0.000	Reference -0.004
9.089	10201	0.043	0.948	15.493	14:0 3OH/16:1 ISO I	6.58	ECL deviates 0.003	Sum In Feature 3
9.616	49408	0.042	0.942	15.818	16:1 CIS 9	31.69	ECL deviates 0.001	
9.908	41775	0.043	0.939	15.999	16:0	26.71	ECL deviates -0.001	Reference -0.005
11.418	17801	0.046	0.927	16.890	17:0 CYCLO	11.23	ECL deviates 0.002	Reference -0.002
13.031	25877	0.045	0.918	17.823	18:1 CIS 11/t 9/t 6	16.17	ECL deviates 0.001	Sum In Feature 7
13.335	2493	0.048	0.917	17.998	18:0	1.56	ECL deviates -0.002	Reference -0.005
14.899	626	0.038	0.917	18.901	19:0 CYCLO C11-12 .	0.39	ECL deviates 0.001	Reference -0.002
18.016	25484	0.049	...	20.716	....	...	> max rt	
*******	10201	...	...	...	SUMMED FEATURE 3 ..	6.58		
*******	25877	...	...	...	SUMMED FEATURE 7 ..	16.17		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30230000	157127	156518	99.61	146928	6	0.004	0.002

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES	SIMILARITY
Alcaligenes	0.305
A. faecalis	0.305
A. denitrificans	0.057
A. d. dentirificans	0.057
Enterobacter	0.052
E. agglomerans	0.052 (Erwinia herbicola)
E. agglomerans biotype II	0.052
Erwinia	0.039
E. salicis	0.039
E. chrysanthemi	0.016
E. c. IV	0.016
E. uredovora	0.004

TUE 31-MAR-87 23:20:29

BOTTLE: 13 ID#: 1403
SAMPLE TYPE: SAMPLE
UN-INGRID 25AD
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

Figure 31: Integrated fatty acid profile of Isolate number 149.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 11
ID: 1401
Name: UN-INGRID 6D **Isolate 149**

Date of report: 31-MAR-87 22:25:17
Date of run: 31-MAR-87 22:25:17

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	29839000	0.060	. . .	7.021	SOLVENT PEAK	. . .	< min rt	
4.091	721	0.028	1.072	11.608	12:0 ISO	1.00	ECL deviates -0.000	Reference -0.006
5.086	7760	0.033	1.028	12.612	13:0 ISO	10.36	ECL deviates 0.000	Reference -0.006
5.183	898	0.035	1.025	12.702	13:0 ANTEISO	1.19	ECL deviates 0.001	Reference -0.005
6.308	4664	0.036	0.994	13.617	14:0 ISO	6.02	ECL deviates -0.001	Reference -0.006
6.806	2404	0.040	0.983	14.000	14:0	3.07	ECL deviates -0.000	Reference -0.005
7.728	25326	0.039	0.967	14.622	15:0 ISO	31.80	ECL deviates 0.001	Reference -0.004
7.862	3698	0.039	0.965	14.712	15:0 ANTEISO	4.63	ECL deviates 0.001	Reference -0.004
9.071	3537	0.040	0.948	15.483	16:1 ISO I/14:0 3OH	4.36	ECL deviates 0.001	Sum In Feature 3
9.301	5440	0.042	0.946	15.625	16:0 ISO	6.68	ECL deviates -0.001	Reference -0.006
9.679	8819	0.044	0.942	15.859	16:1 TRANS 9/15i2OH	10.78	ECL deviates 0.003	Sum In Feature 4
9.907	2878	0.043	0.939	16.000	16:0	3.51	ECL deviates -0.000	Reference -0.006
10.563	1681	0.050	0.933	16.387	17:1 ISO E	2.04	ECL deviates 0.000	
10.688	3678	0.049	0.932	16.461	17:1 ISO H	4.45	ECL deviates -0.000	
10.827	961	0.046	0.931	16.543	17:1 ANTEISO A . . .	1.16	ECL deviates 0.002	
10.973	6401	0.045	0.930	16.629	17:0 ISO	7.73	ECL deviates 0.000	Reference -0.005
11.131	1008	0.046	0.929	16.722	17:0 ANTEISO	1.22	ECL deviates 0.000	Reference -0.005
18.017	24949	0.048	. . .	20.710	. . . .	. . .	> max rt	
*******	3537	. . .	. . .	. . .	SUMMED FEATURE 3 . .	4.36		
*******	8819	. . .	. . .	. . .	SUMMED FEATURE 4 . .	10.78		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
29839000	79874	79874	100.00	77012	11	0.005	0.001

****** QUESTION ANALYSIS RESULTS ******
TOTAL AREA LESS THAN 100000. CONCENTRATE AND RE-RUN.

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES	SIMILARITY	
Bacillus	0.557	
B. thuringiensis	0.556	(B. cereus group)
B. cereus	0.347	
B. mycoides	0.288	(B. cereus group)

TUE 31-MAR-87 22:22:13

BOTTLE: 11 ID#: 1401
SAMPLE TYPE: SAMPLE
UN-INGRID 6D
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

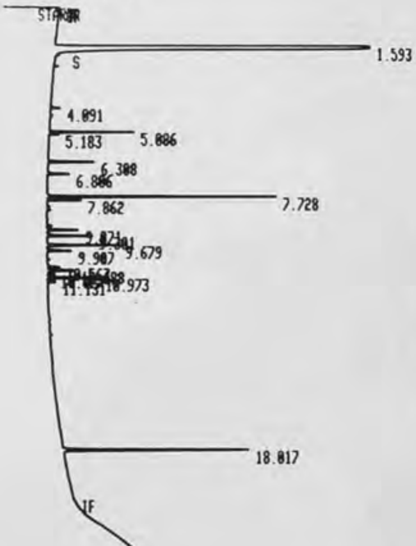


Figure 32: Integrated fatty acid profile of Isolate number 201.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87121591
Bottle: 16
ID: 1187
Name: UM INGRID 25C (BACILLUS) **Isolate 201**

Date of report: 21-JAN-87 23:12:51
Date of run: 21-JAN-87 23:12:51

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.613	38715000	0.077	. . .	7.138	SOLVENT PEAK	. . .	< min rt	
1.800	697	0.018	. . .	7.513	. . . .	. . .	< min rt	
1.833	4859	0.026	. . .	7.580	. . . .	. . .	< min rt	
2.456	605	0.027	. . .	8.855	. . . .	. . .	< min rt	
4.189	1523	0.031	1.084	11.609	12:0 ISO	0.99	ECL deviates 0.001	Reference -0.005
4.530	559	0.032	1.067	11.999	12:0	0.36	ECL deviates -0.001	Reference -0.005
5.202	13939	0.034	1.042	12.612	13:0 ISO	8.74	ECL deviates -0.000	Reference -0.004
5.300	1146	0.034	1.039	12.701	13:0 ANTEISO	0.72	ECL deviates -0.000	Reference -0.003
6.441	6615	0.037	1.007	13.617	14:0 ISO	4.01	ECL deviates -0.001	Reference -0.005
6.946	4857	0.039	0.995	14.000	14:0	2.91	ECL deviates 0.000	Reference -0.004
7.876	39673	0.040	0.977	14.622	15:0 ISO	23.32	ECL deviates 0.001	Reference -0.003
8.011	3326	0.039	0.975	14.713	15:0 ANTEISO	1.95	ECL deviates 0.002	Reference -0.003
9.074	5067	0.043	0.958	15.389	16:1 ISO E	2.92	ECL deviates 0.003	
9.229	2091	0.066	0.956	15.484	16:1 ISO I/14:0 3OH	1.20	ECL deviates 0.002	Sum In Feature 3
9.458	14603	0.046	0.952	15.624	16:0 ISO	8.36	ECL deviates -0.002	Reference -0.007
9.679	6808	0.043	0.949	15.760	16:1 A	3.89	ECL deviates 0.003	
9.778	2024	0.040	0.948	15.821	16:1 CIS 9	1.15	ECL deviates 0.004	
9.842	11146	0.046	0.947	15.860	16:1 TRANS 9/15i2OH	6.35	ECL deviates 0.004	Sum In Feature 4
10.071	11650	0.043	0.944	16.000	16:0	6.62	ECL deviates 0.000	Reference -0.005
10.444	973	0.042	0.940	16.220	15:0 2OH	0.55	ECL deviates 0.003	
10.733	24047	0.047	0.937	16.390	17:1 ISO E	13.55	ECL deviates 0.003	
10.871	4431	0.080	0.935	16.471	17:1 ISO I/ANTEI B .	2.49	ECL deviates -0.005	Sum In Feature 5
11.143	14524	0.044	0.932	16.631	17:0 ISO	8.14	ECL deviates 0.002	Reference -0.003
11.301	1233	0.042	0.931	16.724	17:0 ANTEISO	0.69	ECL deviates 0.002	Reference -0.004
13.098	1154	0.047	0.916	17.762	18:1 CIS 9	0.64	ECL deviates -0.007	
13.511	845	0.046	0.913	17.999	18:0	0.46	ECL deviates -0.001	Reference -0.006
*******	2091	. . .	. . .	. . .	SUMMED FEATURE 3 . .	1.20		
*******	11146	. . .	. . .	. . .	SUMMED FEATURE 4 . .	6.35		
*******	4431	. . .	. . .	. . .	SUMMED FEATURE 5 . .	2.49		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
38715000	172234	172234	100.00	166277	13	0.004	0.003

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES	SIMILARITY
Bacillus	0.180
B. mycoides	0.180 (B. cereus group)

WED 21-JAN-87 23:10:01

BOTTLE: 16 ID#: 1187
SAMPLE TYPE: SAMPLE
UN INGRID 25C (BACILLUS)
FILE NAME: DATA:F87121591

WORKFILE ID: A
WORKFILE NAME:

Figure 33: Integrated fatty acid profile of Isolate number 210.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 24
ID: 1414
Name: UN-INGRID 34bC **Isolate 210**

Date of report: 01-APR-87 05:08:26
Date of run: 01-APR-87 05:08:26

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.589	30319000	0.061	. . .	7.016	SOLVENT PEAK	. . .	< min rt	
2.396	402	0.024	. . .	8.806	. . . .	. . .	< min rt	
2.939	1576	0.026	1.180	10.000	10:0	0.69	ECL deviates 0.000	Reference 0.000
3.323	8415	0.027	1.139	10.605	11:0 ISO	3.55	ECL deviates 0.000	Reference -0.001
3.938	406	0.025	1.091	11.428	10:0 3OH	0.16	ECL deviates 0.005	
4.257	3941	0.032	1.072	11.803	unknown 11.798 . . .	1.56	ECL deviates 0.005	
4.407	790	0.046	. . .	11.980	. . . .	. . .		
4.527	3470	0.031	1.057	12.095	11:0 ISO 3OH	1.36	ECL deviates 0.005	
5.086	1434	0.044	1.034	12.612	13:0 ISO	0.55	ECL deviates -0.000	Reference -0.002
5.184	545	0.035	1.031	12.702	13:0 ANTEISO	0.21	ECL deviates 0.001	Reference -0.000
5.640	1169	0.039	1.015	13.103	12:0 ISO 3OH	0.44	ECL deviates 0.005	
6.105	8719	0.037	1.002	13.460	12:0 3OH	3.23	ECL deviates 0.005	
6.309	5207	0.035	0.996	13.617	14:0 ISO	1.92	ECL deviates -0.001	Reference -0.002
6.808	11511	0.037	0.984	14.000	14:0	4.19	ECL deviates 0.000	Reference -0.001
6.977	7349	0.039	0.981	14.114	13:0 ISO 3OH	2.67	ECL deviates 0.004	
7.102	2232	0.039	0.978	14.198	13:0 2OH	0.81	ECL deviates 0.007	
7.422	3187	0.039	0.972	14.414	15:1 ISO F	1.15	ECL deviates 0.000	
7.731	85443	0.039	0.967	14.622	15:0 ISO	30.58	ECL deviates 0.001	Reference 0.000
7.864	46243	0.039	0.964	14.712	15:0 ANTEISO	16.51	ECL deviates 0.001	Reference 0.000
8.293	2459	0.040	0.957	15.001	15:0	0.87	ECL deviates 0.001	Reference 0.000
9.303	5448	0.041	0.944	15.625	16:0 ISO	1.90	ECL deviates -0.001	Reference -0.002
9.545	9872	0.042	0.941	15.774	16:1 B	3.44	ECL deviates 0.000	
9.616	36856	0.043	0.940	15.818	16:1 CIS 9	12.83	ECL deviates 0.001	
9.909	14276	0.043	0.937	15.999	16:0	4.95	ECL deviates -0.001	Reference -0.002
10.617	8922	0.044	0.931	16.418	17:1 ISO F	3.07	ECL deviates ˙.002	
10.976	5208	0.044	0.928	16.630	17:0 ISO	1.79	ECL deviates 0.001	Reference -0.001
12.939	2828	0.048	0.917	17.770	18:1 CIS 9	0.96	ECL deviates 0.001	
13.033	1767	0.046	0.916	17.824	18:1 TRANS 9/t6/c11	0.60	ECL deviates -0.001	Sum In Feature 7
18.021	27132	0.050	. . .	20.702	. . . .	. . .	> max rt	
*******	1767	. . .	. . .	. . .	SUMMED FEATURE 7 . .	0.60		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30319000	279273	278483	99.72	270125	12	0.001	0.003

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES SIMILARITY

Xanthomonas 0.594
  X. maltophilia . . . . 0.594 (Pseudomonas maltophilia)
  X. campestris 0.123
    X. c. juglans 0.122
    X. c. raphani 0.085
    X. c. cucurbitae 0.085

WED 01-APR-87 05:06:17
,
BOTTLE: 24 ID#: 1414
SAMPLE TYPE: SAMPLE
UN-INGRID 34BC
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

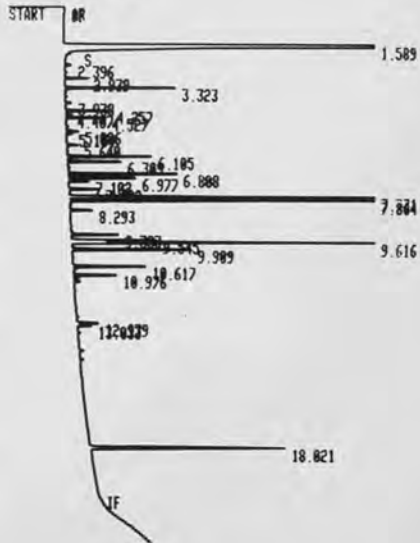


Figure 34: Integrated fatty acid profile of Isolate number 169.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350G12870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 22
ID: 1412
Name: UN-INGRID26aD **Isolate 169**

Date of report: 01-APR-87 04:10:41
Date of run: 01-APR-87 04:10:41

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	30694000	0.061	. . .	7.038	SOLVENT PEAK	. . .	< min rt	
2.396	396	0.024	. . .	8.803	. . . .	. . .	< min rt	
3.523	723	0.034	. . .	10.916	. . . .	. . .		
4.426	3966	0.031	1.062	12.000	12:0	2.83	ECL deviates -0.000	Reference 0.000
6.808	10930	0.043	0.984	14.001	14:0	7.24	ECL deviates 0.001	Reference -0.001
7.554	1025	0.042	0.970	14.503	unknown 14.503 . . .	0.67	ECL deviates 0.000	
8.292	865	0.049	0.957	15.001	15:0	0.56	ECL deviates 0.001	Reference -0.001
9.090	11818	0.042	0.947	15.493	14:0 3OH/16:1 ISO I	7.53	ECL deviates 0.003	Sum In Feature 3
9.616	44459	0.043	0.940	15.818	16:1 CIS 9	28.13	ECL deviates 0.001	
9.910	44588	0.043	0.937	15.999	16:0	28.11	ECL deviates -0.001	Reference -0.001
11.419	11383	0.044	0.925	16.889	17:0 CYCLO	7.08	ECL deviates 0.001	Reference 0.002
13.032	28956	0.046	0.916	17.820	18:1 CIS 11/t 9/t 6	17.85	ECL deviates -0.002	Sum In Feature 7
18.018	28023	0.050	. . .	20.704	. . . .	. . .	> max rt	
18.293	2672	0.145	. . .	20.864	. . . .	. . .	> max rt	
*******	11818	. . .	. . .	. . .	SUMMED FEATURE 3 . .	7.53		
*******	28956	. . .	. . .	. . .	SUMMED FEATURE 7 . .	17.85		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30694000	158713	157990	99.54	148640	5	0.001	0.001

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES SIMILARITY

Klebsiella 0.732
 K. terrigena 0.732
 K. oxytoca 0.199
 K. pneumoniae 0.174
  K. p. ozaenae 0.174
  K. p. pneumoniae 0.103

Enterobacter 0.672
 E. intermedium 0.645
 E. agglomerans . . . . 0.586 (Erwinia herbicola)
  E. agglomerans biotype II 0.586
  E. agglomerans biotype III 0.185
  E. agglomerans biotype I 0.142
 E. cloacae 0.205

Serratia 0.666
 S. plymuthica 0.666
 S. marcescens 0.352
 S. odorifera 0.224

WED 01-APR-87 04:08:35

BOTTLE: 22 ID#: 1412
SAMPLE TYPE: SAMPLE
UN-INGRID26AD
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

Figure 35: Integrated fatty acid profile of Isolate number 174.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 5
ID: 1395
Name: UN-INGRID 31aD   Isolate 174

Date of report: 31-MAR-87 19:33:22
Date of run: 31-MAR-87 19:33:22

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	29600000	0.059	. . .	7.048	SOLVENT PEAK	. . .	< min rt	
3.006	947	0.032	. . .	10.102	. . . .	. . .		
3.526	1202	0.030	1.109	10.919	unknown 10.928 . . .	1.00	ECL deviates -0.009	
4.428	7432	0.031	1.053	12.000	12:0	5.86	ECL deviates 0.000	Reference -0.002
5.089	895	0.032	1.028	12.612	13:0 ISO	0.69	ECL deviates -0.000	Reference -0.003
6.809	3453	0.052	0.983	14.000	14:0	2.54	ECL deviates 0.000	Reference -0.003
7.556	457	0.022	0.970	14.504	unknown 14.503 . . .	0.33	ECL deviates 0.001	
7.729	982	0.035	0.967	14.621	15:0 ISO	0.71	ECL deviates -0.000	Reference -0.004
9.091	7260	0.042	0.948	15.494	14:0 3OH/16:1 ISO I	5.15	ECL deviates 0.004	Sum In Feature 3
9.618	55127	0.042	0.942	15.820	16:1 CIS 9	38.88	ECL deviates 0.003	
9.910	29160	0.043	0.939	16.000	16:0	20.50	ECL deviates 0.000	Reference -0.004
10.148	1258	0.044	0.937	16.141	15:0 ISO 3OH	0.88	ECL deviates 0.006	
10.617	2423	0.043	0.933	16.418	17:1 ISO F	1.69	ECL deviates 0.002	
10.975	2427	0.044	0.930	16.629	17:0 ISO	1.69	ECL deviates -0.000	Reference -0.004
11.254	789	0.042	0.928	16.793	17:1 B	0.55	ECL deviates 0.001	
11.604	710	0.044	0.925	17.000	17:0	0.49	ECL deviates 0.000	Reference -0.004
13.032	27704	0.045	0.918	17.825	18:1 TRANS 9/t6/c11	19.04	ECL deviates -0.000	Sum In Feature 7
18.018	23615	0.047	. . .	20.720	. . . .	. . .	> max rt	
*******	7260	. . .	. . .	. . .	SUMMED FEATURE 3 . .	5.15		
*******	27704	. . .	. . .	. . .	SUMMED FEATURE 7 . .	19.04		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
29600000	142226	141279	99.33	133620	7	0.004	0.003

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES	SIMILARITY
Aeromonas	0.360
A. hydrophila	0.360
Neisseria	0.031
N. sicca	0.031 (48 hrs)
Pleisomonas	0.017
P. shigelloides	0.017

TUE 31-MAR-87 19:27:25

BOTTLE: 5 ID#: 1395
SAMPLE TYPE: SAMPLE
UN-INGRID 31AD
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

Figure 36: Integrated fatty acid profile of Isolate number 222.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 17
ID: 1407
Name: UN-INGRID 3cY **Isolate 222**

Date of report: 01-APR-87 01:46:33
Date of run: 01-APR-87 01:46:33

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	30561000	0.061	. . .	7.108	SOLVENT PEAK	. . .	< min rt	
4.029	992	0.031	1.085	11.536	unknown 11.541 . . .	0.42	ECL deviates -0.005	
5.088	571	0.032	1.034	12.612	13:0 ISO	0.23	ECL deviates 0.000	Reference -0.000
5.431	910	0.039	1.021	12.929	13:1 AT 12-13 . . .	0.36	ECL deviates -0.002	
6.231	2703	0.039	0.998	13.556	unknown 13.566 . . .	1.06	ECL deviates -0.010	
6.807	1688	0.036	0.984	13.999	14:0	0.65	ECL deviates -0.001	Reference -0.002
7.459	8631	0.044	0.971	14.439	15:1 ISO G	3.29	ECL deviates -0.002	
7.729	68366	0.039	0.967	14.621	15:0 ISO	25.94	ECL deviates 0.000	Reference -0.001
7.863	4930	0.038	0.964	14.712	15:0 ANTEISO	1.87	ECL deviates 0.001	Reference -0.001
8.079	11410	0.041	0.961	14.857	15:1 B	4.30	ECL deviates 0.001	
8.292	20077	0.040	0.957	15.001	15:0	7.54	ECL deviates 0.001	Reference -0.001
9.033	795	0.039	0.947	15.459	16:1 ISO H	0.30	ECL deviates -0.002	
9.089	1338	0.046	0.947	15.494	14:0 3OH/16:1 ISO I	0.50	ECL deviates 0.004	Sum In Feature 3
9.301	2318	0.049	0.944	15.625	16:0 ISO	0.86	ECL deviates -0.001	Reference -0.003
9.616	37188	0.045	0.940	15.820	16:1 CIS 9	13.73	ECL deviates 0.003	
9.764	846	0.047	0.939	15.911	16:1 C	0.31	ECL deviates 0.003	
9.908	10105	0.043	0.937	16.000	16:0	3.72	ECL deviates 0.000	Reference -0.002
10.144	20893	0.045	0.935	16.140	15:0 ISO 3OH	7.67	ECL deviates 0.005	
10.296	779	0.044	. . .	16.230	. . . .	. . .		
10.614	10280	0.044	0.931	16.417	17:1 ISO F	3.76	ECL deviates 0.001	
10.765	6510	0.076	0.930	16.507	15:0 3OH	2.37	ECL deviates 0.003	
10.899	2582	0.052	0.928	16.586	unknown 16.580 . . .	0.94	ECL deviates 0.006	
10.973	1374	0.042	0.928	16.629	17:0 ISO	0.50	ECL deviates 0.000	Reference -0.002
11.254	2690	0.045	0.926	16.795	17:1 B	0.98	ECL deviates 0.003	
11.373	12388	0.045	0.925	16.866	17:1 C	4.50	ECL deviates 0.004	
11.871	5345	0.052	. . .	17.156	. . . .	. . .		
12.514	10043	0.048	0.918	17.526	16:0 3OH	3.62	ECL deviates 0.006	
12.997	981	0.052	. . .	17.804	. . . .	. . .		
13.625	26581	0.048	0.915	18.166	17:0 ISO 3OH	9.54	ECL deviates 0.002	
13.791	1111	0.060	. . .	18.261	. . . .	. . .		
14.030	952	0.050	0.914	18.399	TBSA	0.34	ECL deviates 0.007	
14.274	1970	0.050	0.914	18.539	17:0 3OH	0.71	ECL deviates 0.004	
18.018	26549	0.050	. . .	20.700	. . . .	. . .	> max rt	
19.657	12897	0.719	. . .	21.642	. . . .	. . .	> max rt	
*******	1338	. . .	. . .	. . .	SUMMED FEATURE 3 . .	0.50		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30561000	277347	269131	97.04	254793	8	0.002	0.004

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES SIMILARITY

Cytophaga 0.044
C. johnsonae 0.044

WED 01-APR-87 01:44:20

BOTTLE: 17 ID#: 1407
SAMPLE TYPE: SAMPLE
UN-INGRID 3CY
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

Figure 37: Integrated fatty acid profile of Isolate number 155.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 19
ID: 1409
Name: UN-INGRID 12aD **Isolate 155**

Date of report: 01-APR-87 02:44:13
Date of run: 01-APR-87 02:44:1.

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	30277000	0.060	. . .	7.019	SOLVENT PEAK	. . .	< min rt	
2.395	396	0.023	. . .	8.788	. . . .	. . .	< min rt	
6.808	5384	0.039	0.984	14.000	14:0	5.08	ECL deviates -0.000	Reference -0.001
8.291	4139	0.039	0.957	15.000	15:0	3.80	ECL deviates 0.000	Reference -0.001
9.614	3225	0.041	0.940	15.818	16:1 CIS 9	2.91	ECL deviates 0.001	
9.680	14504	0.045	0.940	15.859	16:1 TRANS 9/15i2OH	13.07	ECL deviates 0.003	Sum In Feature 4
9.908	25772	0.042	0.937	16.000	16:0	23.16	ECL deviates -0.000	Reference -0.002
11.253	5496	0.049	0.926	16.794	17:1 B	4.88	ECL deviates 0.002	
11.605	1668	0.045	0.923	17.002	17:0	1.48	ECL deviates 0.002	Reference 0.000
12.313	1967	0.044	0.919	17.410	18:1 ISO F	1.73	ECL deviates -0.000	
12.939	26506	0.047	0.917	17.770	18:1 CIS 9	23.29	ECL deviates 0.001	
13.335	1935	0.049	0.915	17.998	18:0	1.70	ECL deviates -0.002	Reference -0.002
14.017	13915	0.047	0.914	18.391	TBSA	12.19	ECL deviates -0.001	
14.653	1390	0.063	0.913	18.757	unknown 18.756/19:1	1.22	ECL deviates 0.001	Sum In Feature 8
15.073	3676	0.052	0.913	18.999	19:0	3.22	ECL deviates -0.001	Reference 0.001
16.796	2596	0.060	0.918	20.001	20:0	2.28	ECL deviates 0.001	Reference 0.006
18.020	27314	0.050	. . .	20.712	. . . .	. . .	> max rt	
18.234	1967	0.051	. . .	20.836	. . . .	. . .	> max rt	
18.498	6298	0.054	. . .	20.989	. . . .	. . .	> max rt	
19.736	331	0.031	. . .	21.707	. . . .	. . .	> max rt	
19.847	1606	0.054	. . .	21.772	. . . .	. . .	> max rt	
*******	14504	. . .	. . .	. . .	SUMMED FEATURE 4 . .	13.07		
*******	1390	. . .	. . .	. . .	SUMMED FEATURE 8 . .	1.22		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30277000	112173	112173	100.00	104312	7	0.003	0.001

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES	SIMILARITY	
Rhodococcus	0.237	
R. rhodochrous	0.237	
R. fascians	0.024	(Corynebacterium fascians)

WED 01-APR-87 02:41:33

BOTTLE: 19 ID#: 1409
SAMPLE TYPE: SAMPLE
UN-INGRID 12AD
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

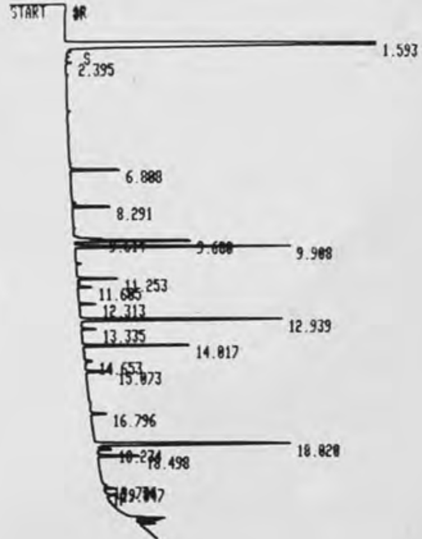


Figure 38: Integrated fatty acid profile of Isolate number 15.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650
Bottle: 20
ID: 1410
Name: UN-INGRID E3 Isolate 15

Date of report: 01-APR-87 03:13:04
Date of run: 01-APR-87 03:13:04

RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	30534000	0.061	. . .	7.054	SOLVENT PEAK	. . .	< min rt	
3.939	3932	0.031	1.091	11.428	10:0 3OH	4.25	ECL deviates 0.005	
4.427	3785	0.031	1.062	12.001	12:0	3.98	ECL deviates 0.001	Reference 0.001
5.743	1898	0.037	1.012	13.181	12:0 2OH	1.90	ECL deviates 0.003	
6.808	612	0.038	0.984	13.999	14:0	0.60	ECL deviates -0.001	Reference -0.001
9.617	52225	0.042	0.940	15.818	16:1 CIS 9	48.68	ECL deviates 0.001	
9.909	34021	0.043	0.937	15.998	16:0	31.60	ECL deviates -0.002	Reference -0.002
11.418	1839	0.044	0.925	16.890	17:0 CYCLO	1.69	ECL deviates 0.002	Reference 0.002
13.031	8039	0.048	0.916	17.823	18:1 CIS 11/t 9/t 6	7.30	ECL deviates 0.001	Sum In Feature 7
18.021	25388	0.049	. . .	20.721	. . . .	. . .	> max rt	
*******	8039	. . .	. . .	. . .	SUMMED FEATURE 7 . .	7.30		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
30534000	106351	106351	100.00	100900	4	0.001	0.002

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES SIMILARITY

Janthinobacterium 0.948
  J. lividum 0.948

Pseudomonas 0.118
  P. putida 0.118
    P. p. biovar B 0.118
  P. acidovorans 0.034
  P. syringae 0.022
    P. s. savastanoi ash 0.022
    P. s. morsprunorum 0.014
    P. s. phaseolicola 0.004

Chromobacterium 0.070
  C. violaceum 0.070

WED 01-APR-87 03:10:26

BOTTLE: 20 ID#: 1410
SAMPLE TYPE: SAMPLE
UN-INGRID E3
FILE NAME: DATA:F87331650

WORKFILE ID: A
WORKFILE NAME:

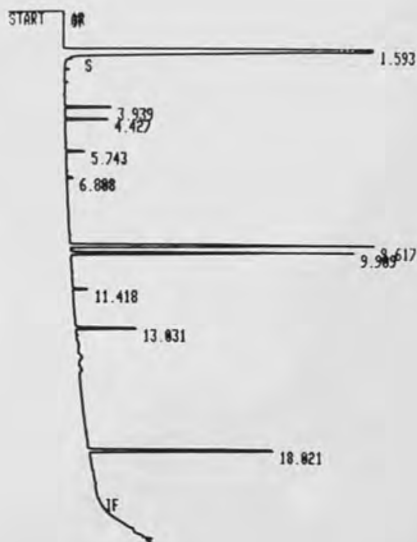


Figure 39: Integrated fatty acid profile of Isolate number 193.

HP 5898A: Microbial Identification System (Vers: 1.2) (Software s/n: 2615A100032 Computer s/n: 2350612870)

*** SAMPLE ***

File: DATA:F87331650 | Date of report: 01-APR-87 00:48:52
Bottle: 15 | Date of run: 01-APR-87 00:48:52
ID: 1405
Name: UN-INGRID MC Isolate 193

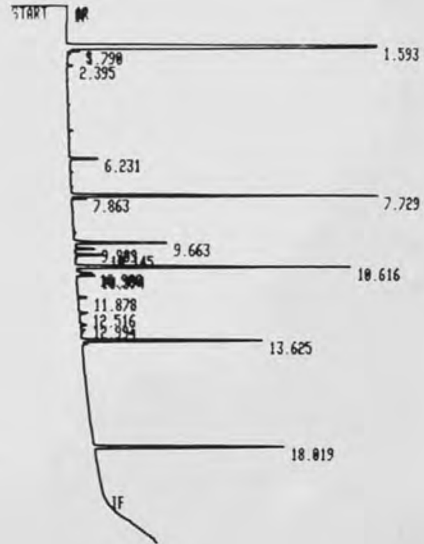
RT	Area	Ar/Ht	Respon	ECL	Name	Area %	Comment 1	Comment 2
1.593	29773000	0.059	. . .	7.052	SOLVENT PEAK	. . .	< min rt	
1.790	542	0.020	. . .	7.483	. . . .	. . .	< min rt	
2.395	378	0.024	. . .	8.808	. . . .	. . .	< min rt	
6.231	2824	0.037	0.998	13.556	unknown 13.566 . . .	2.26	ECL deviates -0.010	
7.729	46430	0.039	0.967	14.621	15:0 ISO	35.92	ECL deviates 0.000	Reference -0.001
7.863	1490	0.037	0.964	14.711	15:0 ANTEISO	1.15	ECL deviates 0.000	Reference -0.001
9.663	12390	0.048	0.940	15.848	15:0 ISO 2OH/16:1t9	9.32	ECL deviates 0.005	Sum In Feature 4
9.909	2361	0.044	0.937	16.000	16:0	1.77	ECL deviates -0.000	Reference -0.002
10.145	3599	0.045	0.935	16.139	15:0 ISO 3OH	2.69	ECL deviates 0.004	
10.616	33208	0.043	0.931	16.418	17:1 ISO F	24.74	ECL deviates 0.002	
10.900	2196	0.051	0.928	16.585	unknown 16.580 . . .	1.63	ECL deviates 0.005	
10.974	2095	0.042	0.928	16.629	17:0 ISO	1.56	ECL deviates 0.000	Reference -0.002
11.878	1243	0.049	. . .	17.160	. . . .	. . .		
12.516	1592	0.063	0.918	17.528	16:0 3OH	1.17	ECL deviates 0.008	
12.994	1036	0.063	. . .	17.803	. . . .	. . .		
13.625	24313	0.048	0.915	18.168	17:0 ISO 3OH	17.80	ECL deviates 0.004	
18.019	25889	0.049	. . .	20.723	. . . .	. . .	> max rt	
*******	12390	. . .	. . .	. . .	SUMMED FEATURE 4 . .	9.32		

Solvent Ar	Total Area	Named Area	% Named	Total Amnt	Nbr Ref	Ref ECL Shift	ECL Deviation
29773000	134777	132498	98.31	124954	4	0.001	0.005

HP 5898A: MIS Aerobic Library (Vers: 1.1) (Library s/n: 2615A100032)

MOST LIKELY MATCHES | SIMILARITY

NO MATCHING LIBRARY ENTRY FOUND



Once organisms had been isolated they were routinely grown at 25°C a temperature at which most grew within 24 hours. Isolates selected for further work were also tested for growth on NA at 4°C over a period of 11 days (table 23).

Isolate	Growth
Xanthomonas	-
Ps. cepacia/chloraphis	+++
Aeromonas	+++
Ps. maltophilia	+++
Alcaligenes	++
Ps. testosteroni	++
Ps. putida	+++
Erwinia	-
Bacillus (spreading)	+
Bacillus (discrete)	-
Rhodococcus	++
Ps. fluorescens	+++
Cytophaga	+++
Proteus	-
Janthinobacterium	+++
Streptomyces (grey)	-
Streptomyces (white)	-

Table 23: Growth of isolates on NA at 4°C over 11 days

- = no growth; + = sparse growth;

++ = good growth; +++ = luxuriant growth

In order that the morphotypes of bacteria isolated and characterised from alder leaves could be related to morphotypes observed *in vivo*, pure cultures of isolates selected for further work were allowed to colonise sterile alder leaf discs which were then prepared and

viewed under SEM. All isolates were rod shapes of varying dimensions, either singly, in pairs or in chains (table 24).

Isolate	Dimensions µm	Distinguishing characteristics
Xanthomonas	1,0 x 0,8	polysaccharide production
Bacillus (discrete)	2,5 x 1,5	chains
Erwinia	1,5 x 1,0	polysaccharide production
Ps. testosteroni	0,9 x 0,7	pairs
Janthinobacterium	1,2 x 0,9	polysaccharide production
Ps.cepacia/ chloraphis	1,2 x 0,8	
Ps.fluorescens	0,4 x 0.7	
Rhodococcus		fragmented rods

Table 24: Dimensions, arrangement and distinguishing characteristics of some isolates selected for further work after colonisation of sterile alder leaf discs as observed under SEM

Polysaccharide was produced by some isolates. Xanthomonas (fig. 40) and Erwinia (fig. 41) appeared to be more active producers of polysaccharide. A small

Figure 40: *Xanthomonas* isolate after 5 days colonisation of sterile alder leaf discs.

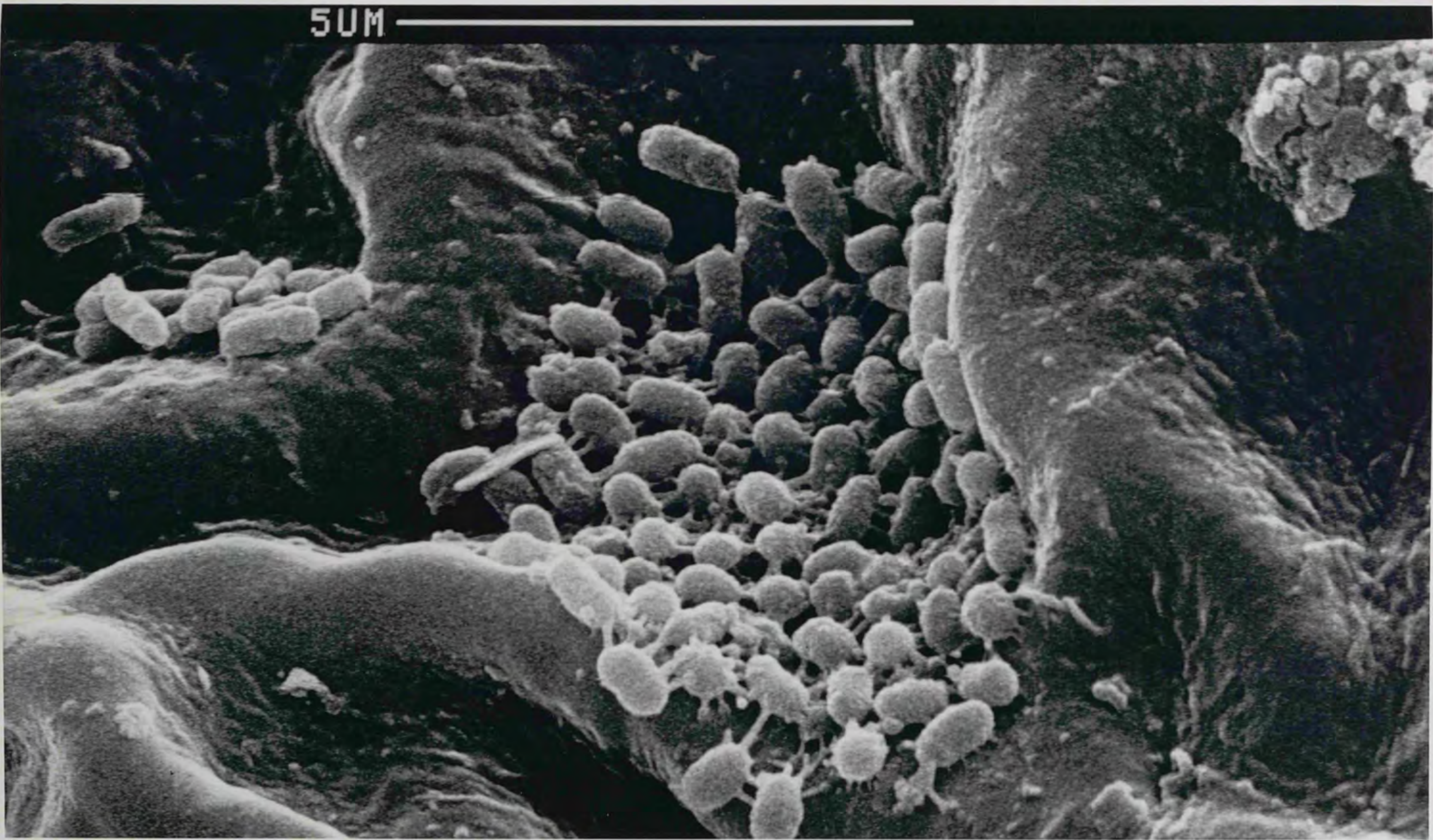
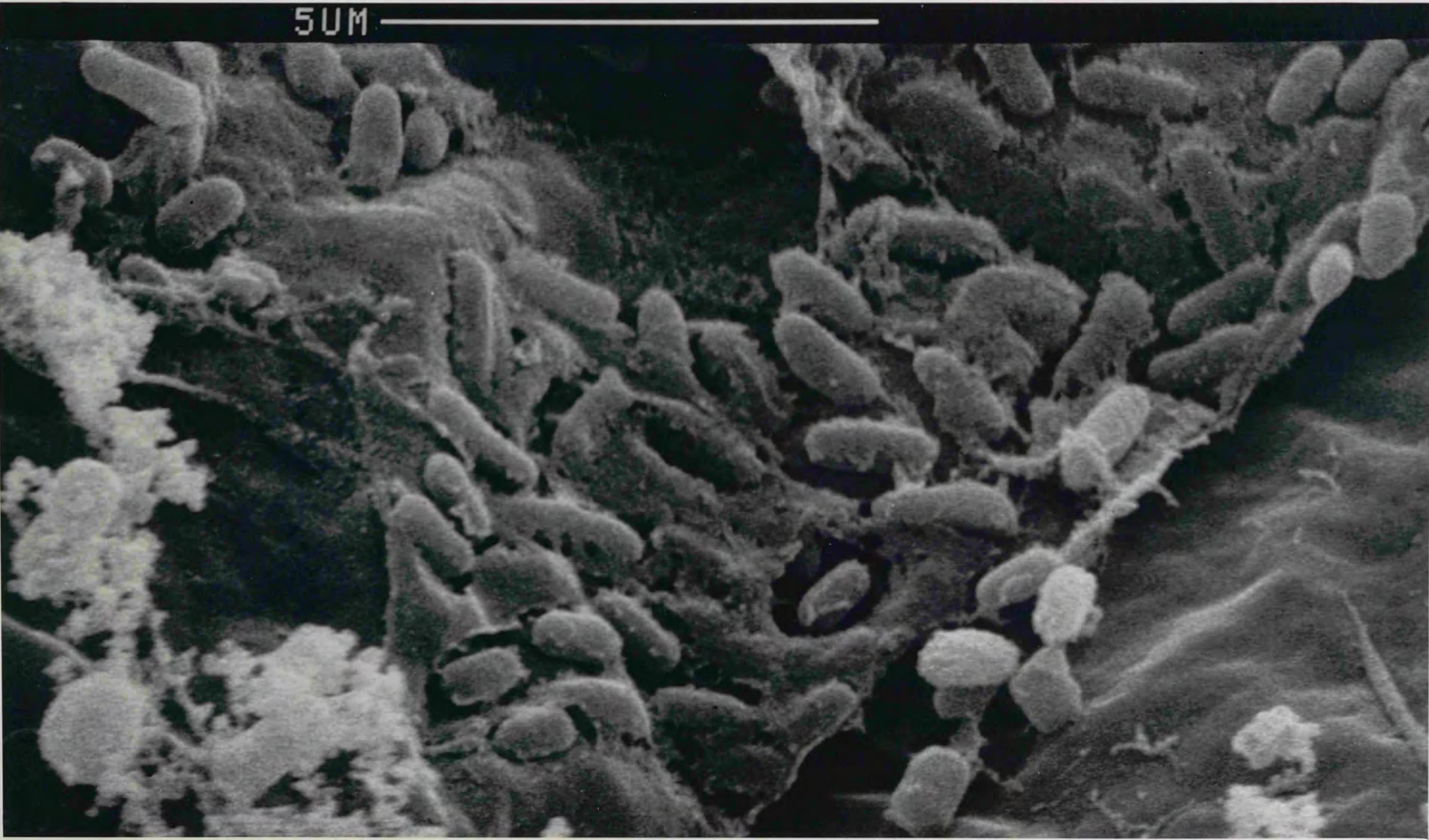

5UM

Figure 41: *Erwinia* isolate after 5 days colonisation of sterile alder leaf discs.


5UM

amount of polysaccharide was also produced by Janthinobacterium (fig. 42). Chains of Bacillus (discrete growth fig. 43) bore a resemblance to the brick-shaped bacilli observed in vivo and the pairs of Ps. testosteroni (fig. 44) isolates were also similar to the pairs of cocco-bacilli observed on in vivo samples. Rhodococcus (fig 45) appeared fragmented after 5 days incubation.

4. In vitro colonisation of alder leaves by a known mixture of bacteria

A succession of morphotypes on leaves incubated with a mixture of microorganisms consisting of Bacillus, Pseudomonas, Aeromonas, Klebsiella, Alcaligenes, Rhodococcus, Xanthomonas, Streptomyces and Erwinia was demonstrated using SEM (figs. 46-49). Three different morphotypes were observed and these were cocco-bacilli, intermediate length bacilli and branched hyphae of bacterial dimensions.

The experimental flasks were inoculated with sterilised leaves. It was considered that such leaves were devoid of yeast cells normally present on the surfaces of dried alder leaves and indeed yeasts cells were not observed, even on the earliest samples. Organisms colonised in a four-stage process:

Initial colonisation (fig. 46)

Cocco-bacilli colonised the alder leaves first and were

Figure 42: *Janthinobacterium* isolate after 5 days colonisation of sterile alder leaf discs.

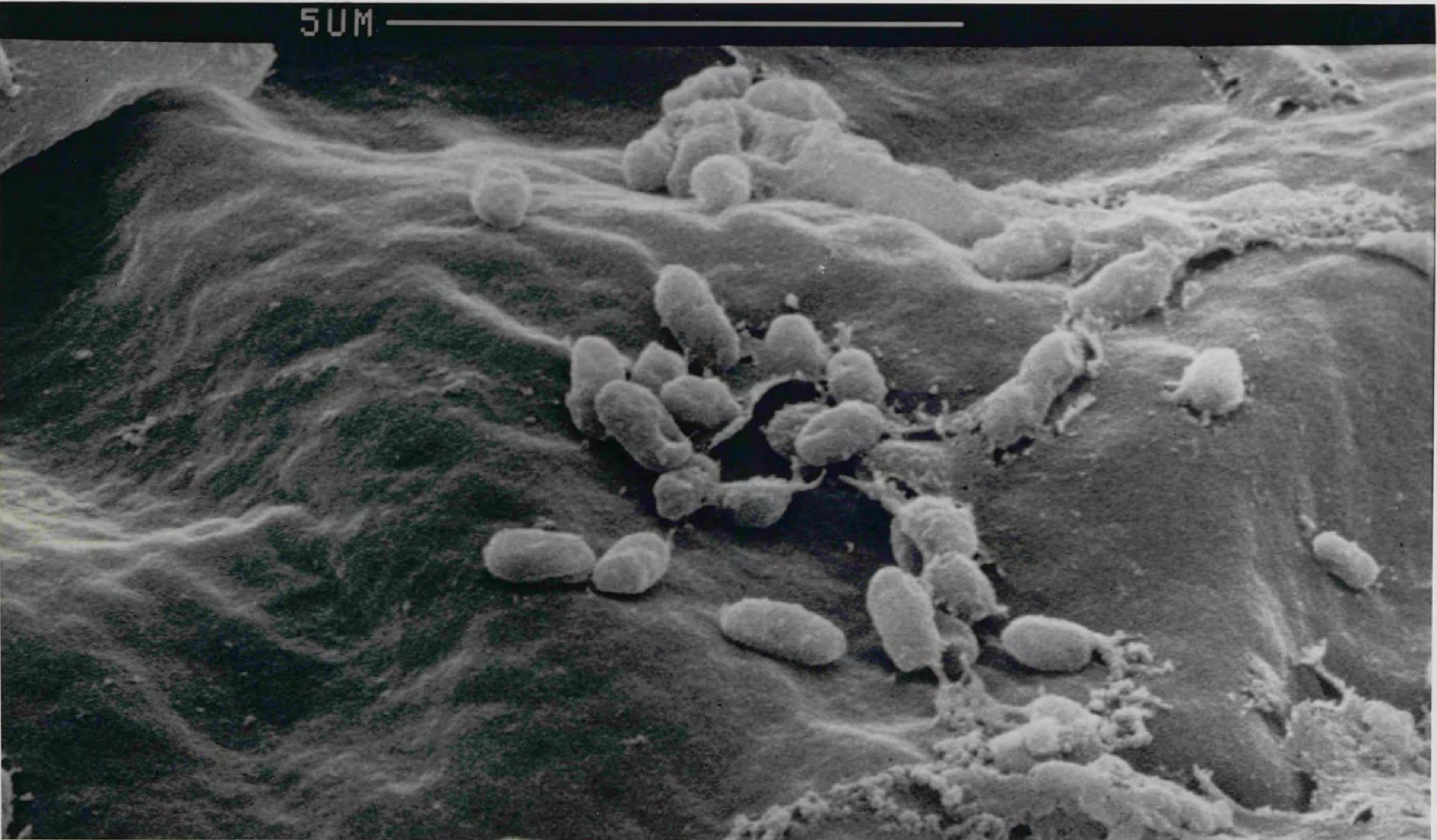
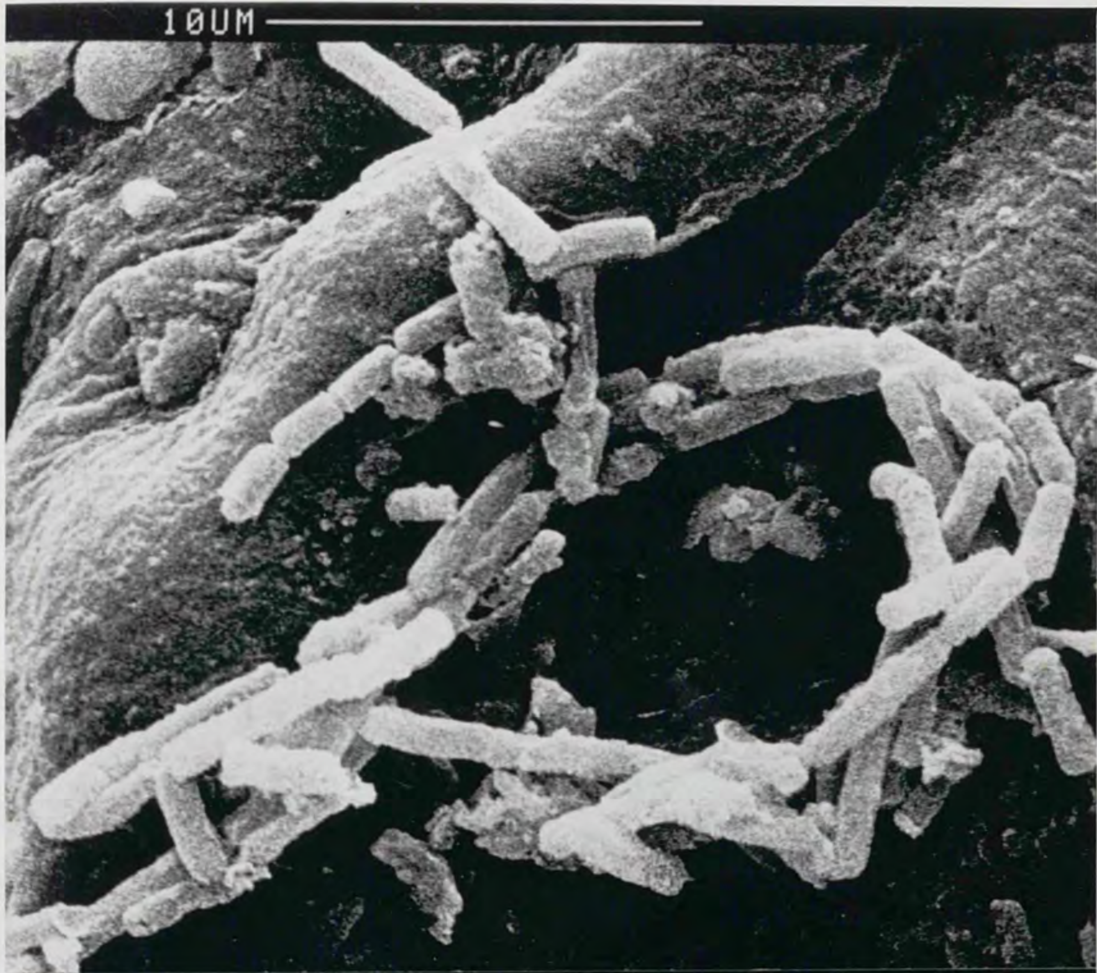

5UM

Figure 43: *Bacillus* isolate after 5 days colonisation of sterile alder leaf discs.


10UM

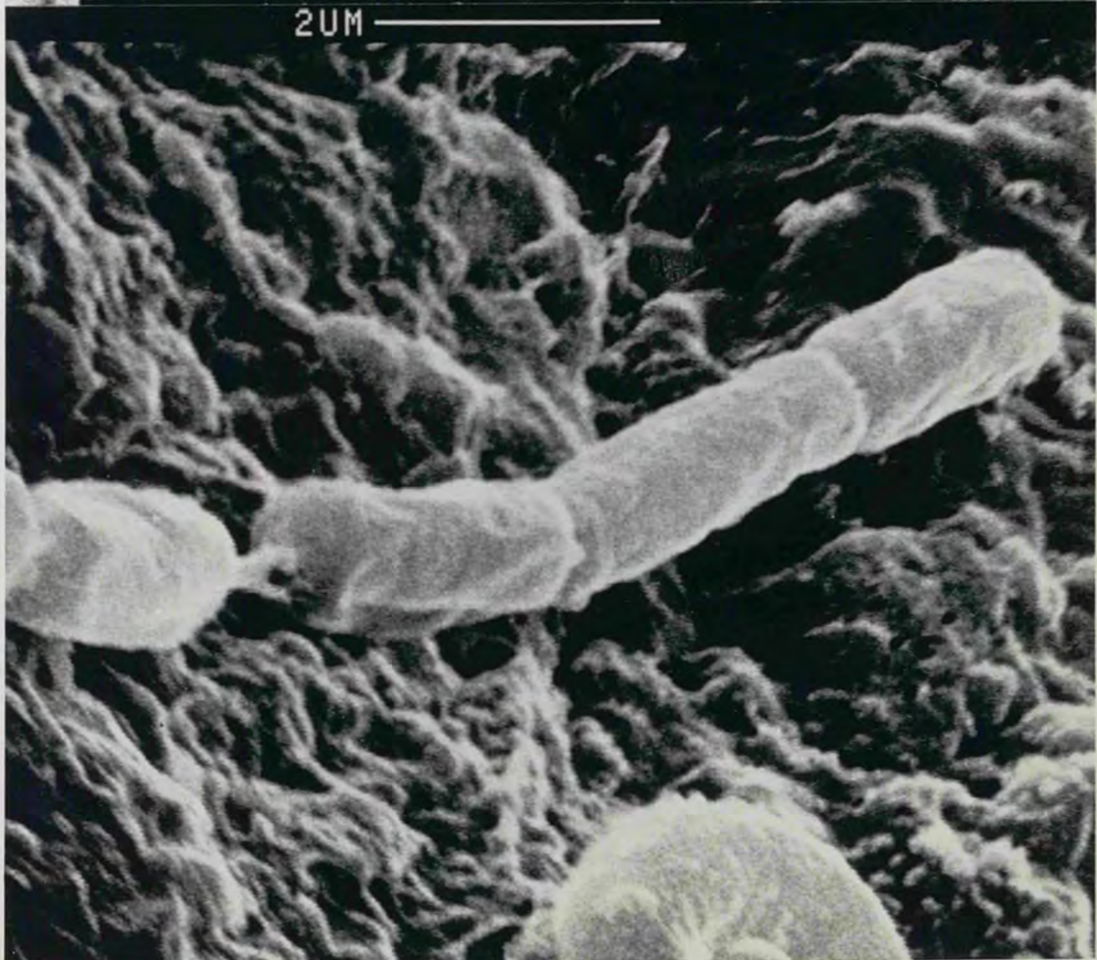

2UM

Figure 44: *Pseudomonas testosteroni* after 5 days colonisation of sterile alder leaf discs.

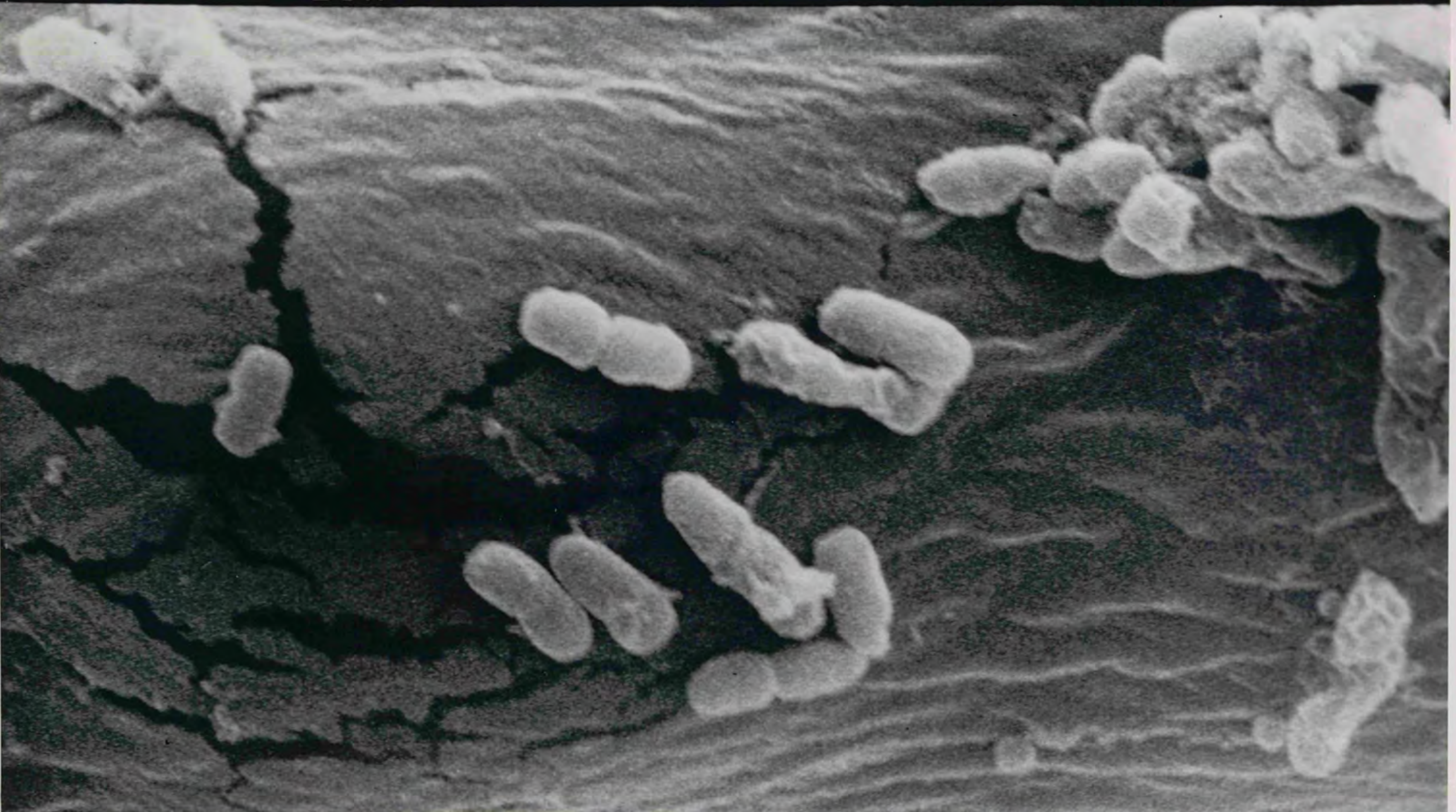

2UM

Figure 45: *Rhodococcus* isolate after 5 days colonisation of sterile alder leaf discs. Bar measurements a, b=10μm.

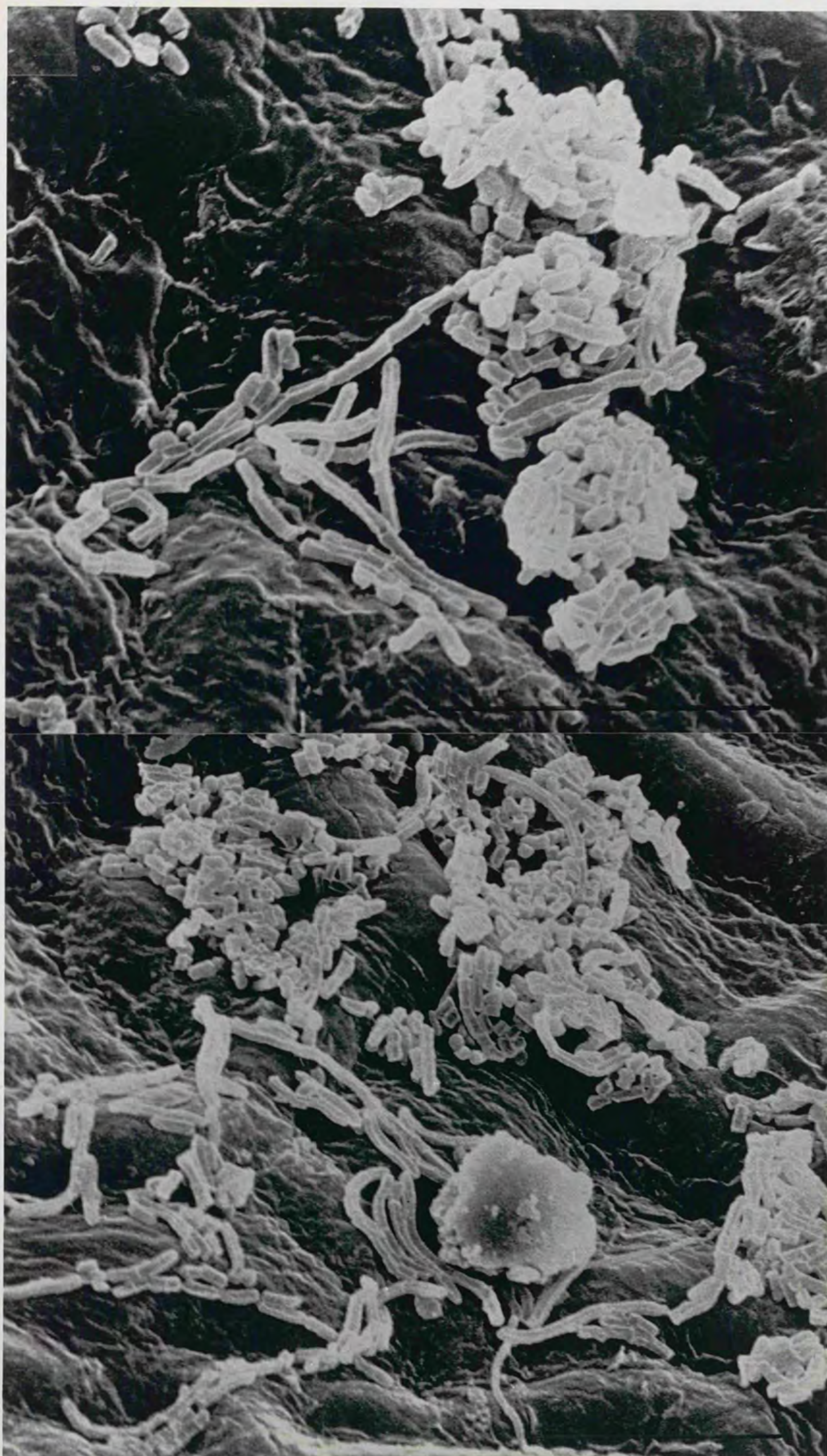
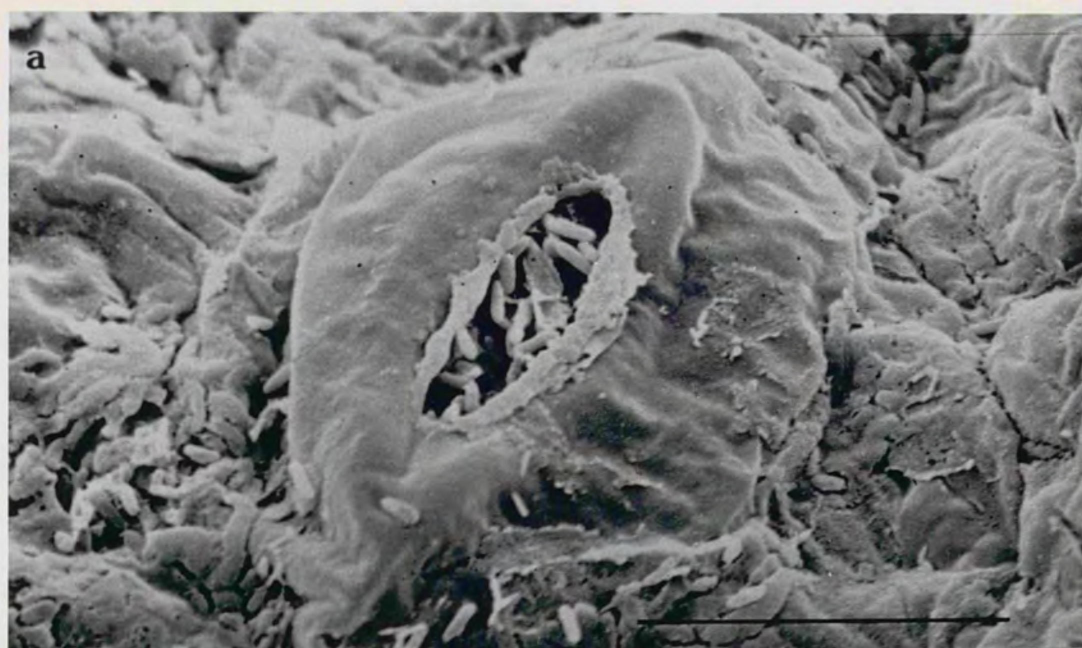
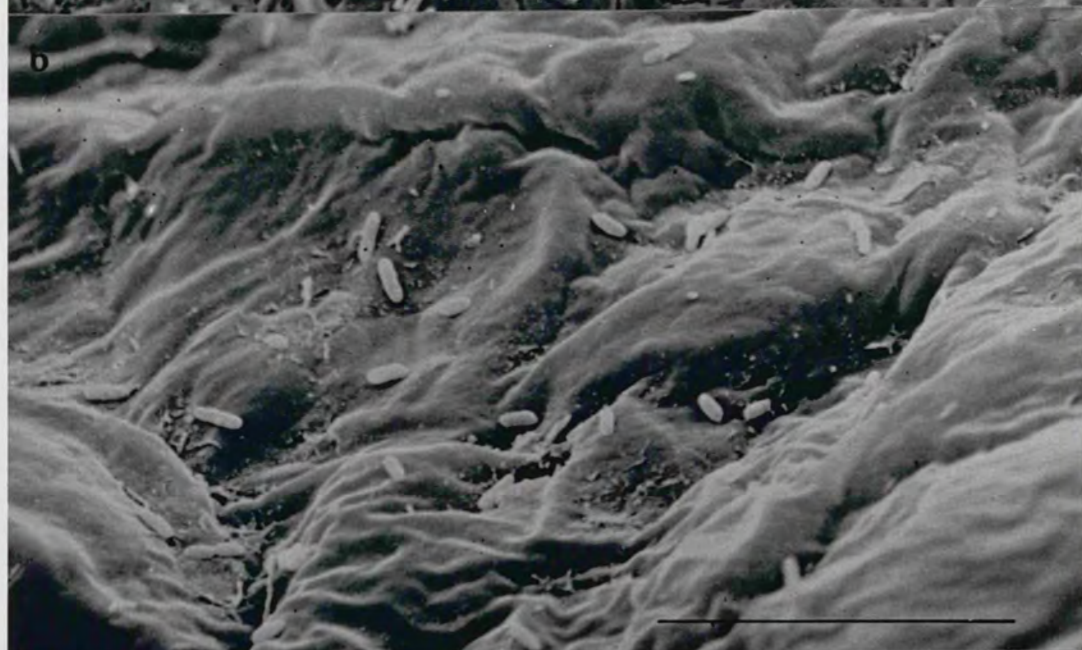


Figure 46: Initial colonisation of sterile alder leaves *in vitro* by a known mixture of microorganisms. Figure 46a shows coccobacilli in and around an open stoma, 46b the typical density of covering by microorganisms after 48h incubation, and 46c the association of coccobacilli with small amounts of polysaccharide. Bar measurements a, b=10μm, c=5μm.


a


b

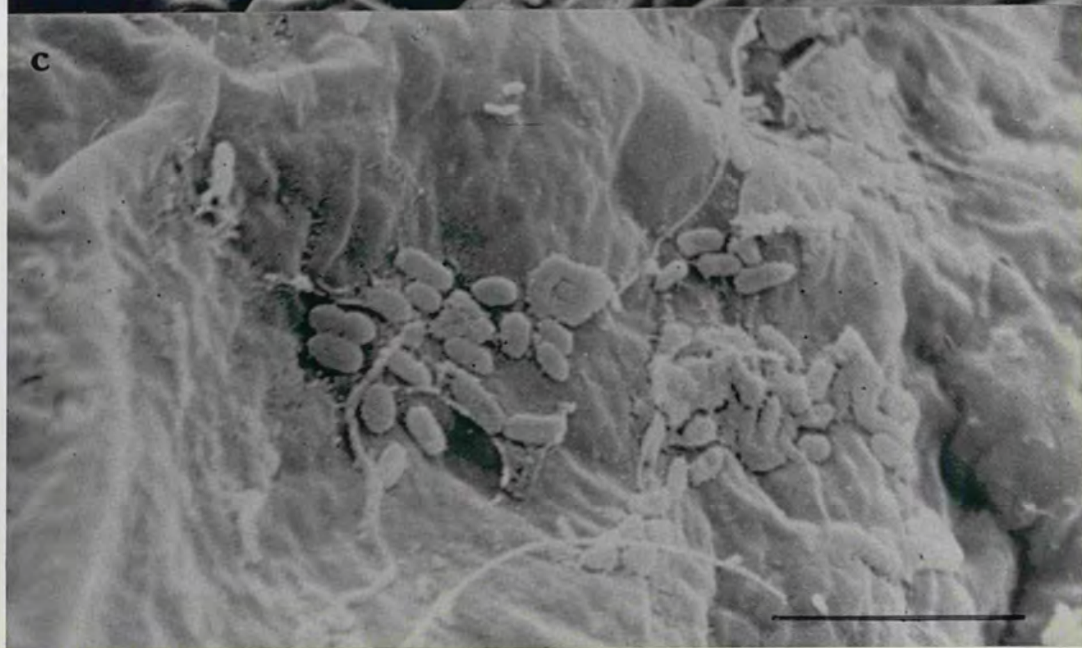
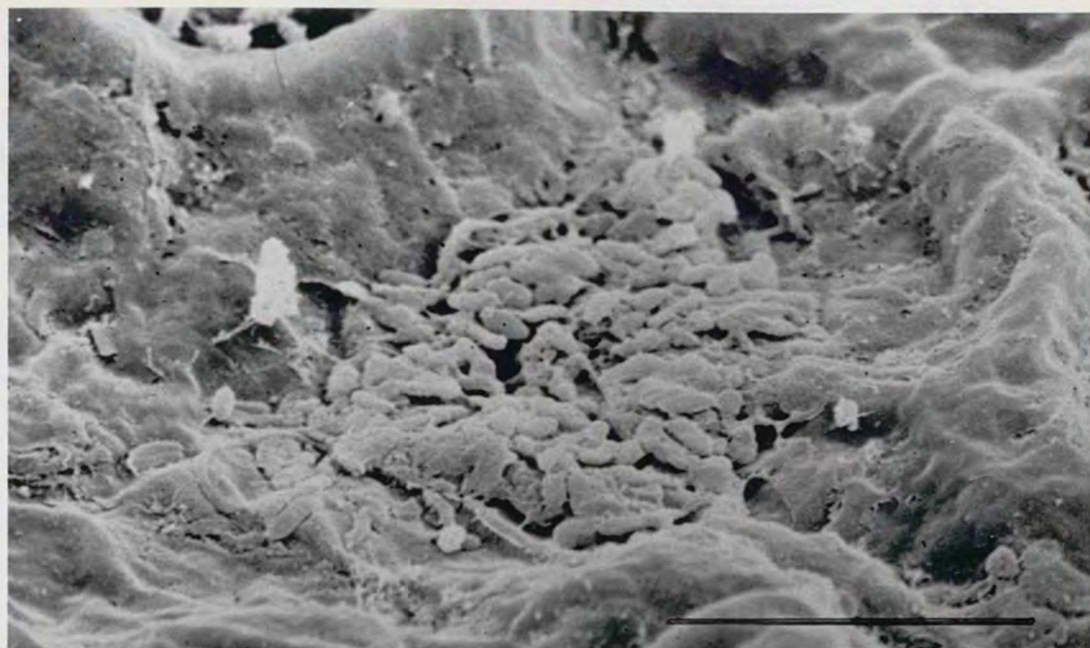
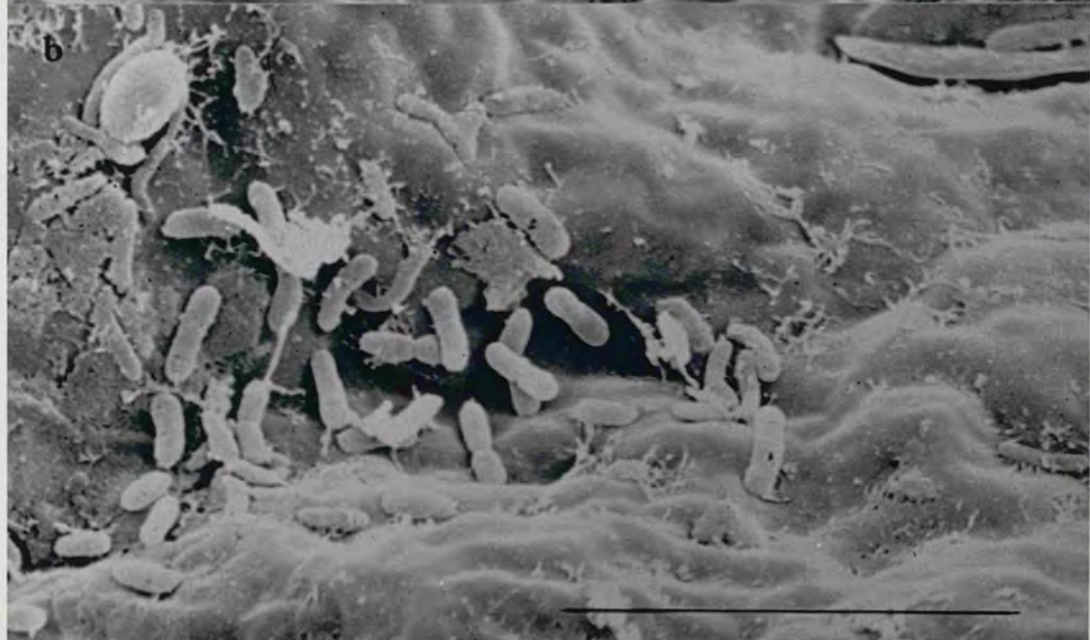

c

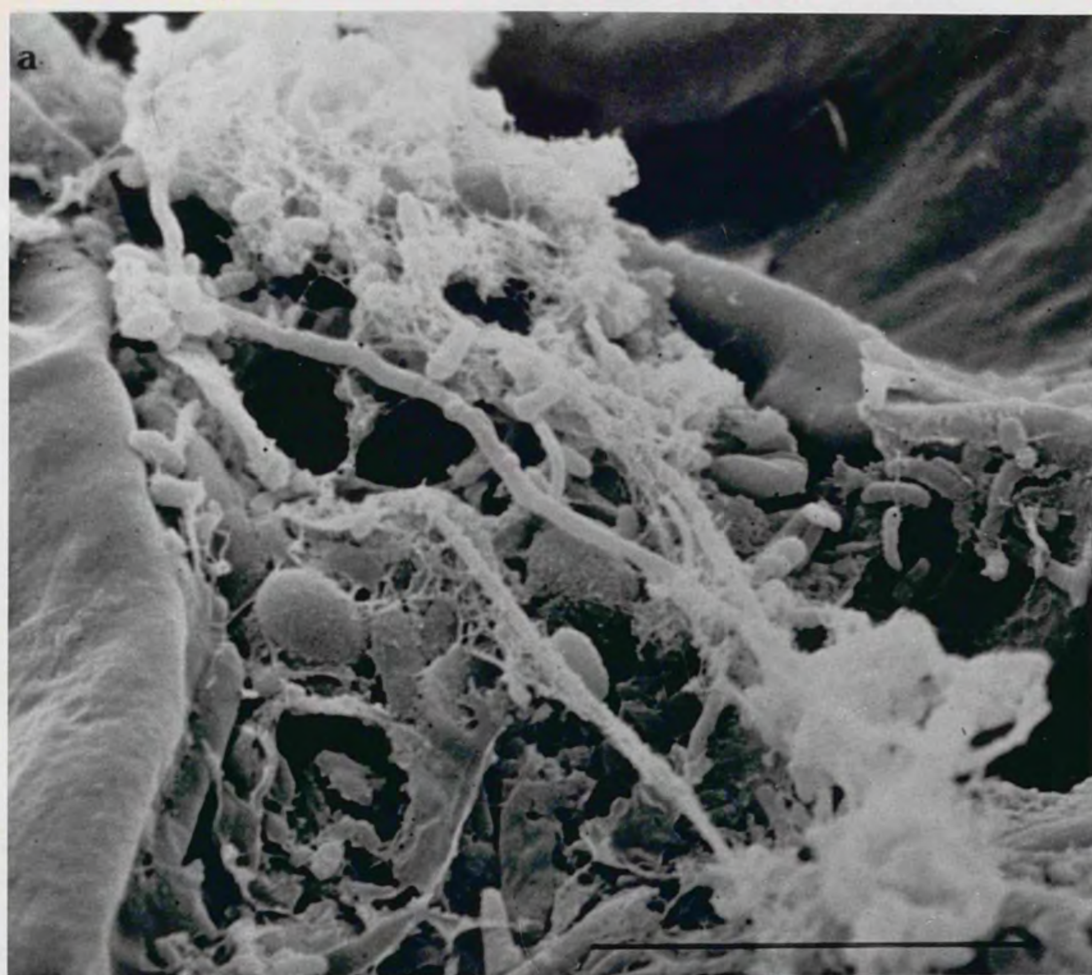
Figure 47: Diversification and proliferation of microorganisms from a known mixture colonising sterile alder leaves *in vitro*. Figure 48a shows a typical larger group of coccobacilli covered by a thick layer of polysaccharide, 47b shows coccobacilli in pairs and 48c intermediate length bacilli. Bar measurements a=20μm, b, c=10μm.




b

c

Figure 48 Diversification and reduction of microorganisms from a known mixture colonising sterile alder leaves *in vitro*. Figure 48a shows typical filamentous, branched hyphae, coccobacilli and intermediate length bacilli, and 48b a typical density of covering of coccobacilli and intermediate length bacilli, both in association with polysaccharide. Bar measurements a=10μm, b=5μm.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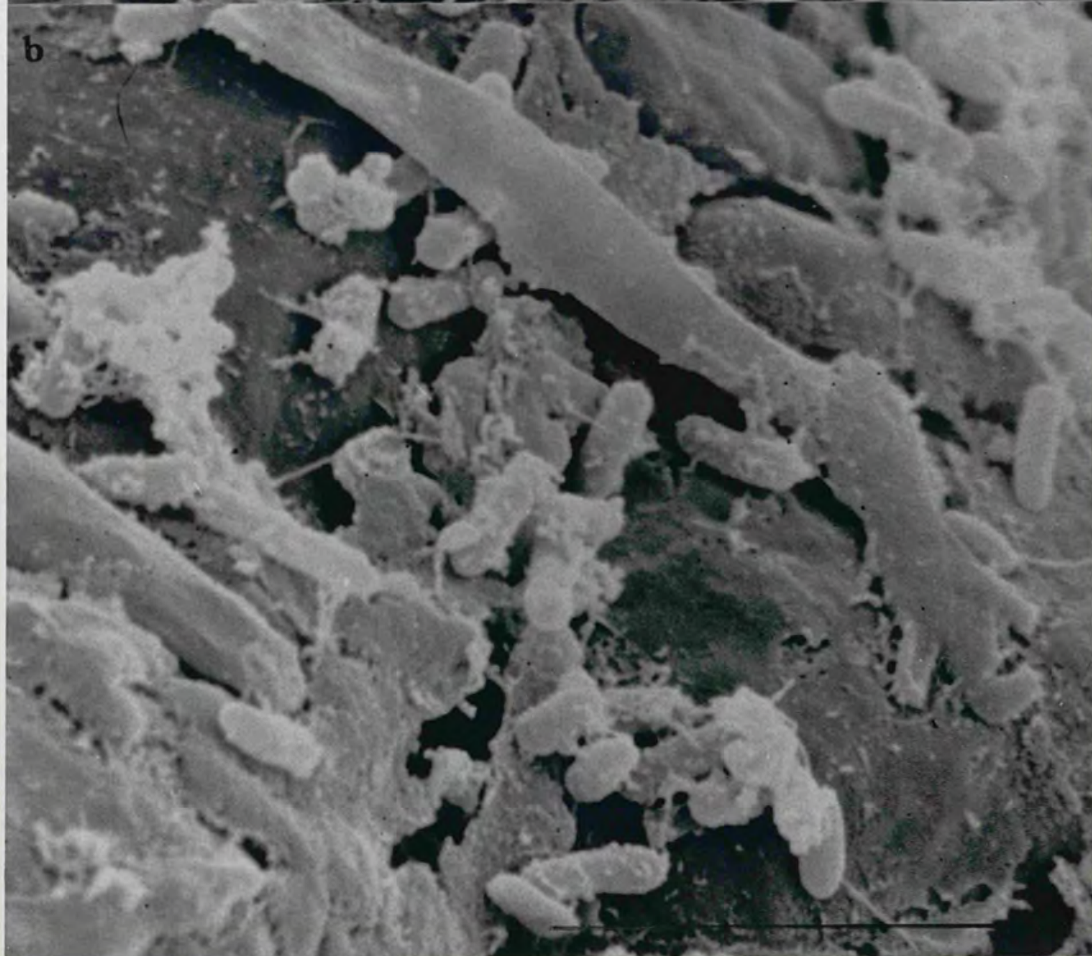
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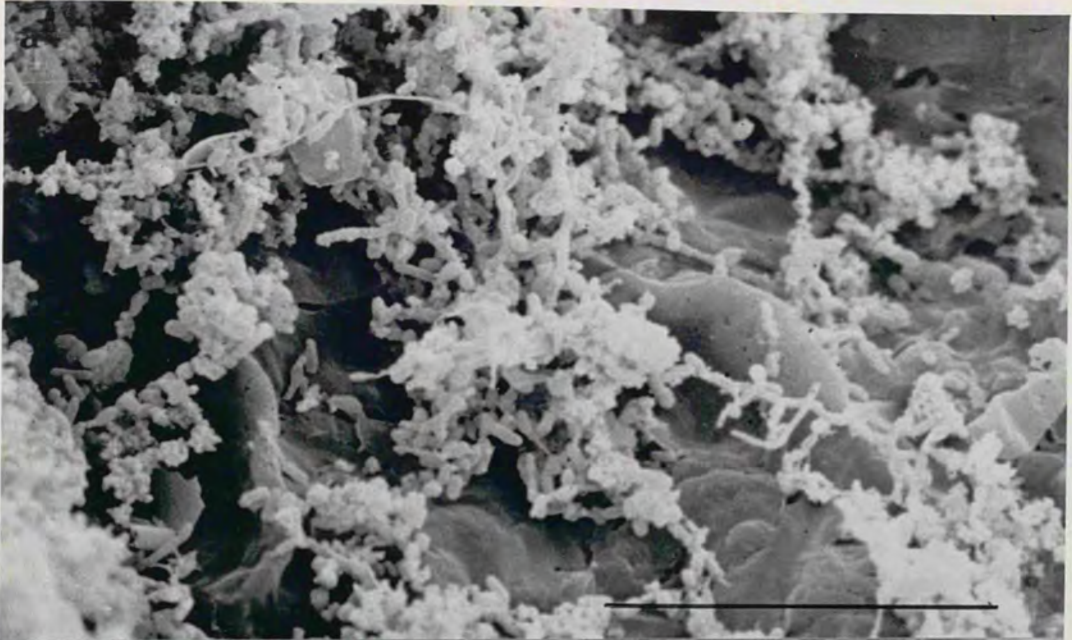
Figure 49: Stabilisation of microorgamisms from a known mixture colonising sterile alder leaves *in vitro*. Figure 49a shows typical density of covering of microrganisms and also some debri, 49b shows the detail of a branched hypha and coccobacilli, and 49c shows branched hyphae. Bar measurements a=20μm, b, c=10μm.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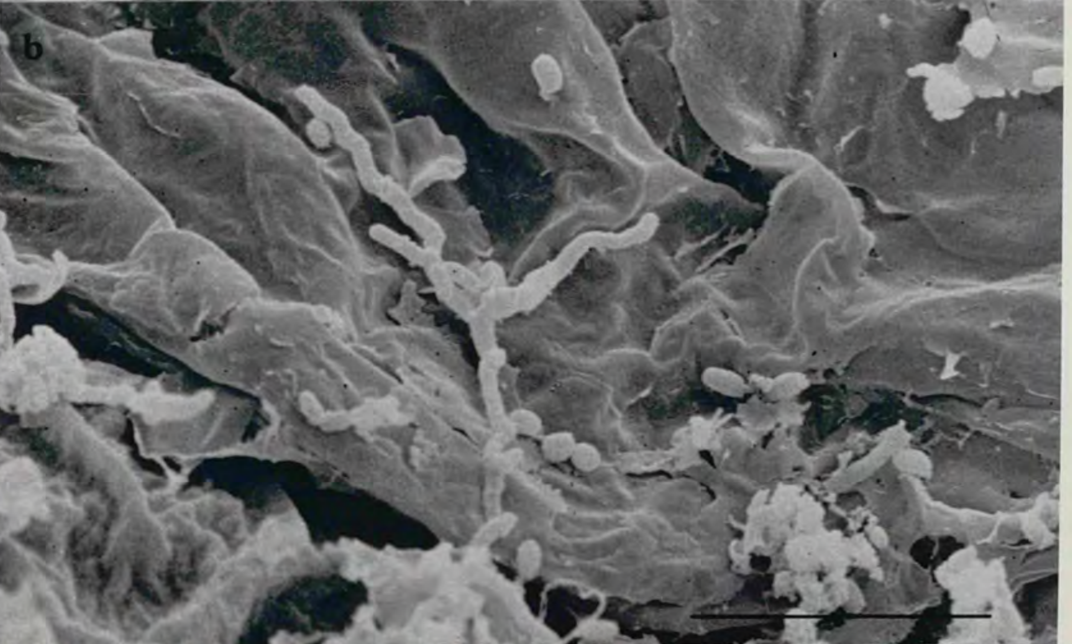
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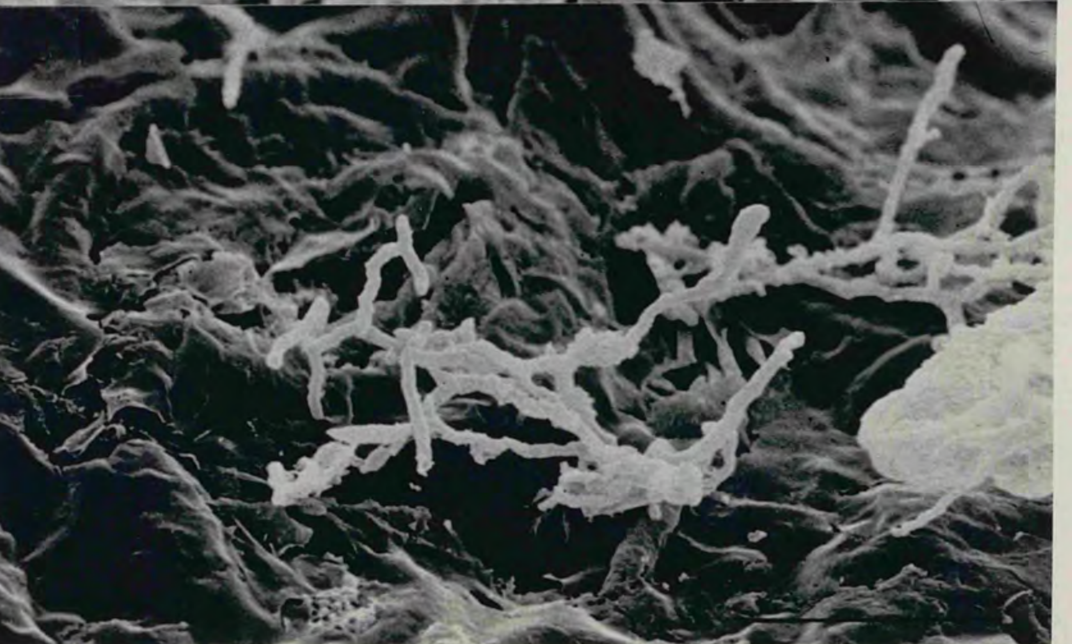


the only morphotype observed after 24 hours incubation. During the first 24 hours cocco-bacilli were observed in isolation, in small groups and clustered in an around stomata. Some groups were associated with small amounts of polysaccharide.

Diversification and proliferation (fig. 47)

Cocco-bacilli increased in number and groups became heavily encrusted with polysaccharide. Intermediate length bacilli colonised after 48 hours incubation and increased in abundance over the week which followed. Intermediate length bacilli were never observed to be the most abundant morphotype, but remained persistent throughout the decay period.

Diversification and reduction (fig. 48)

Cocco-bacilli decreased in abundance and branched hyphae of bacterial dimension were observed. Cocco-bacilli and intermediate length bacilli were observed in association with polysaccharide.

Stabilisation (fig. 49)

Branched hyphae became established and also the most abundant morphotype after approximately two weeks incubation and remained prominent until the end of the 6-week experimental run. Cocco-bacilli and intermediate-length bacilli remained.

Enumeration of colonised organisms by removal and

plating onto selective media showed no consistent pattern of colonisation by the genera inoculated as shown in figs. 50-54.

5. Microscopy - General Observations

SEM preparations from the field study and *in vitro* work showed evidence of burrowing into the leaves by bacteria and also the formation of erosion pits (figs. 55 & 56). Burrow holes appeared after 3 weeks incubation *in vivo* and *in vitro* samples.

Organisms with spirochaete morphology were observed on the leaf surface on one occasion during the field study under SEM (fig. 57). A single observation was not considered sufficient to draw any conclusions from, but they were frequently observed on fresh preparations stained with acridine orange (fig. 58) and in all cases were highly motile. In view of the motility of the organisms with spirochaete-morphology it is likely that they would have been washed off the leaf surface during SEM preparation and therefore infrequently observed on SEM preparations.

6. Enzyme Survey

A survey of the plant cell wall degrading enzymes produced by isolates from degrading alder leaves was undertaken in order to establish whether organisms were able to attack the structural components of plant cell walls.

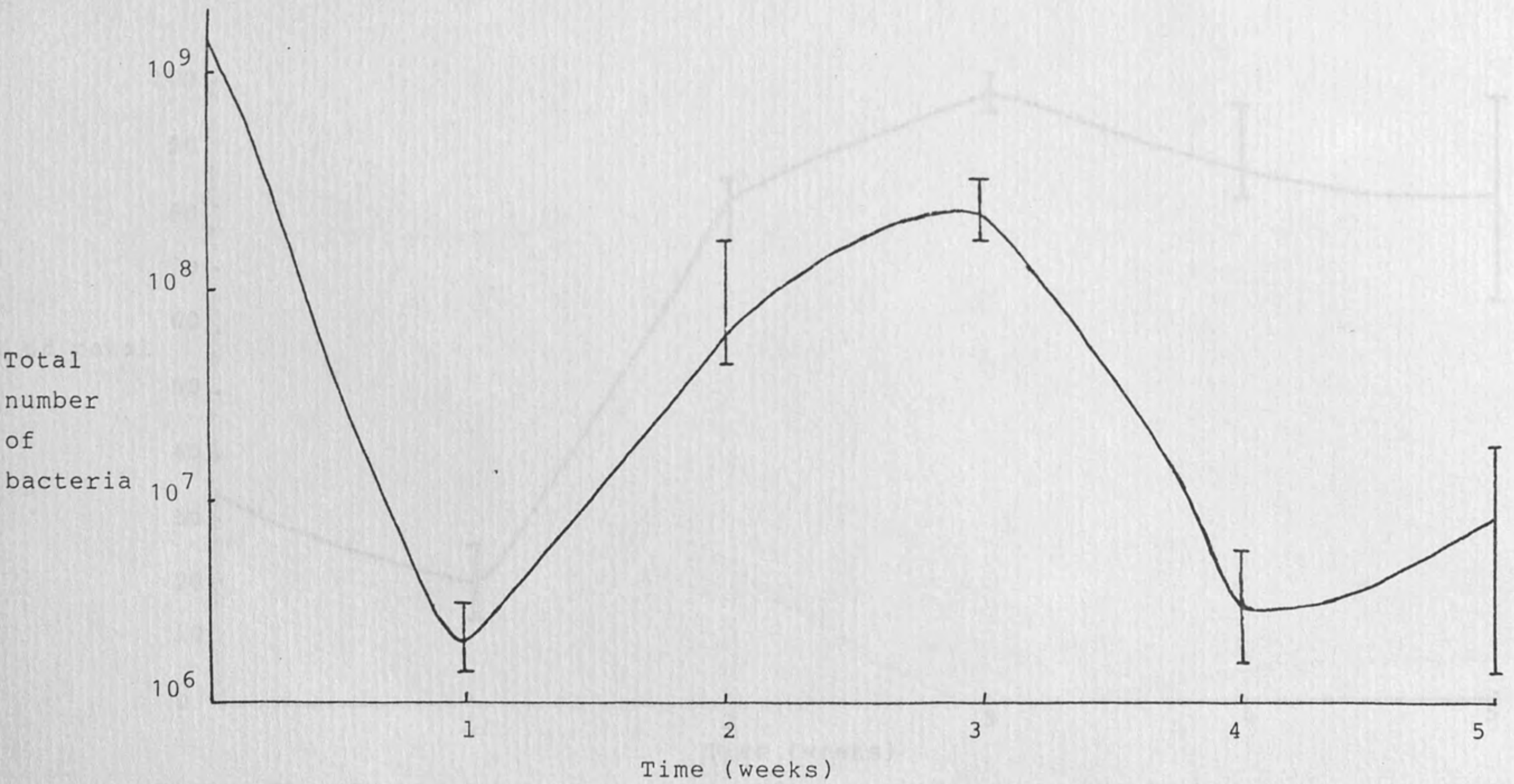


Figure 50: Change in total number of bacteria, cultured on NA, colonising sterile alder leaf discs *in vitro*.

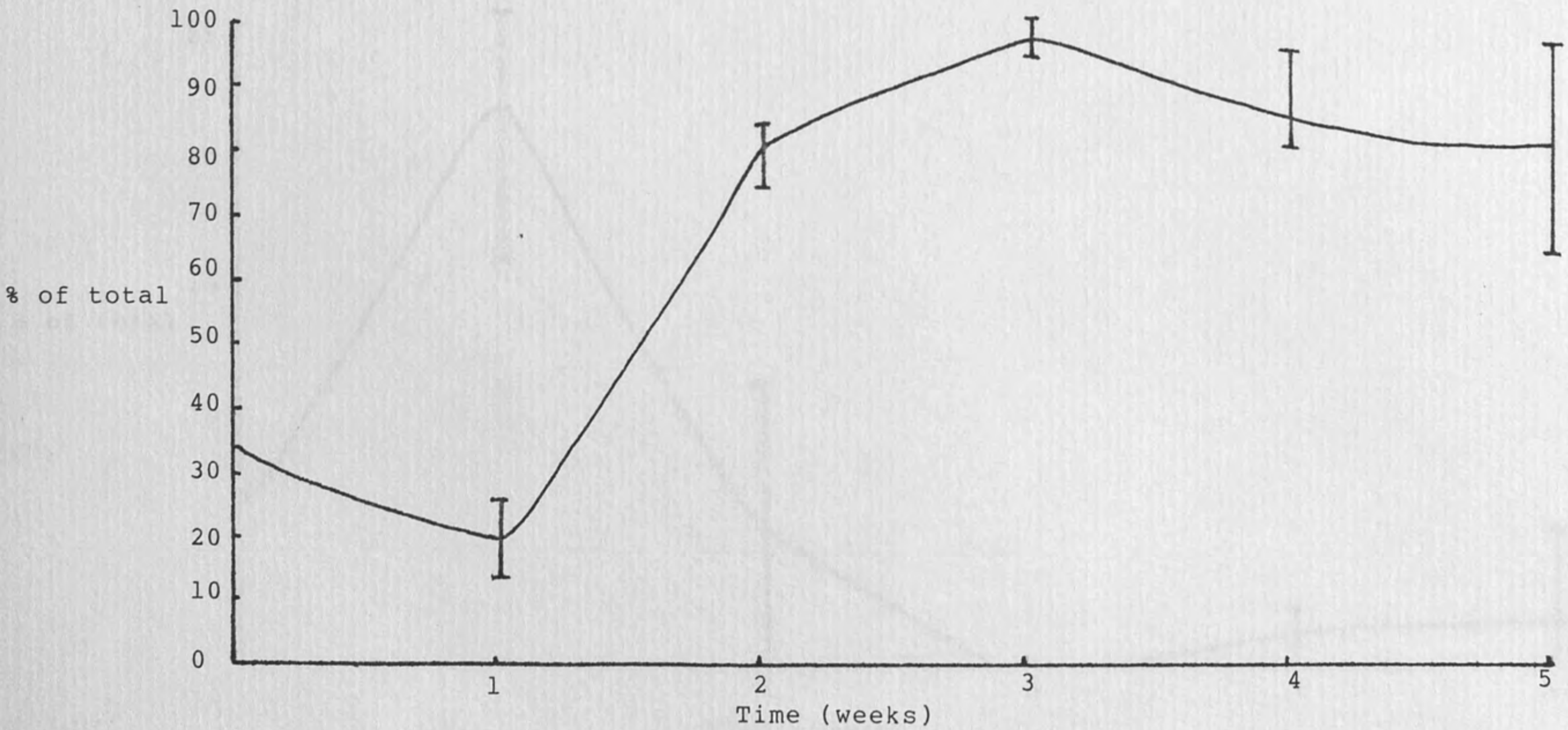


Figure 51: Change in number of *Alcaligenes*, cultured on MacConkey agar as a percentage of the total number of colonies cultured on NA, colonising sterile alder leaf discs *in vitro*.

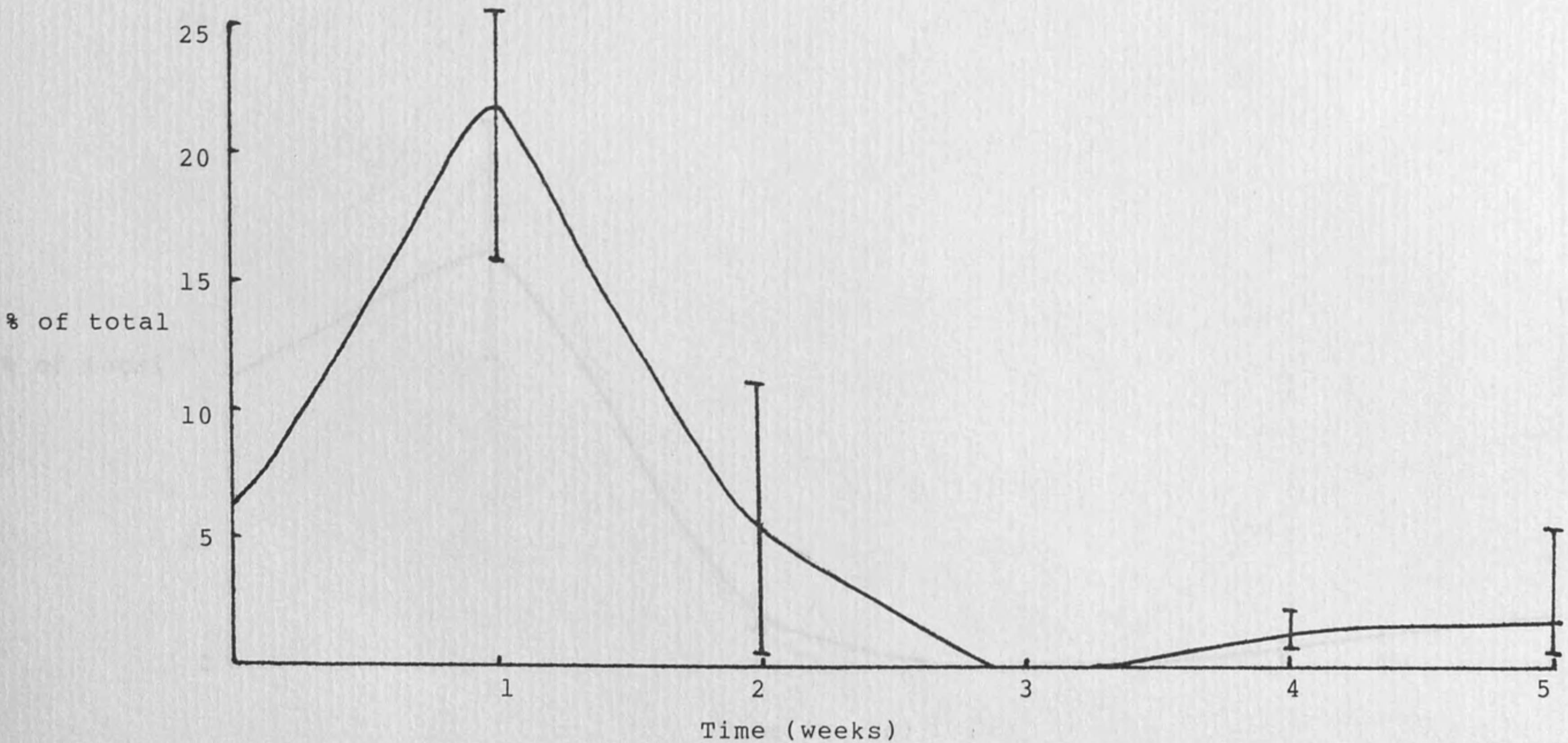


Figure 52: Change in number of *Pseudomonas fluorescens*, cultured on PAA as a percentage of the total number of colonies cultured on NA, colonising sterile alder leaves *in vitro*.

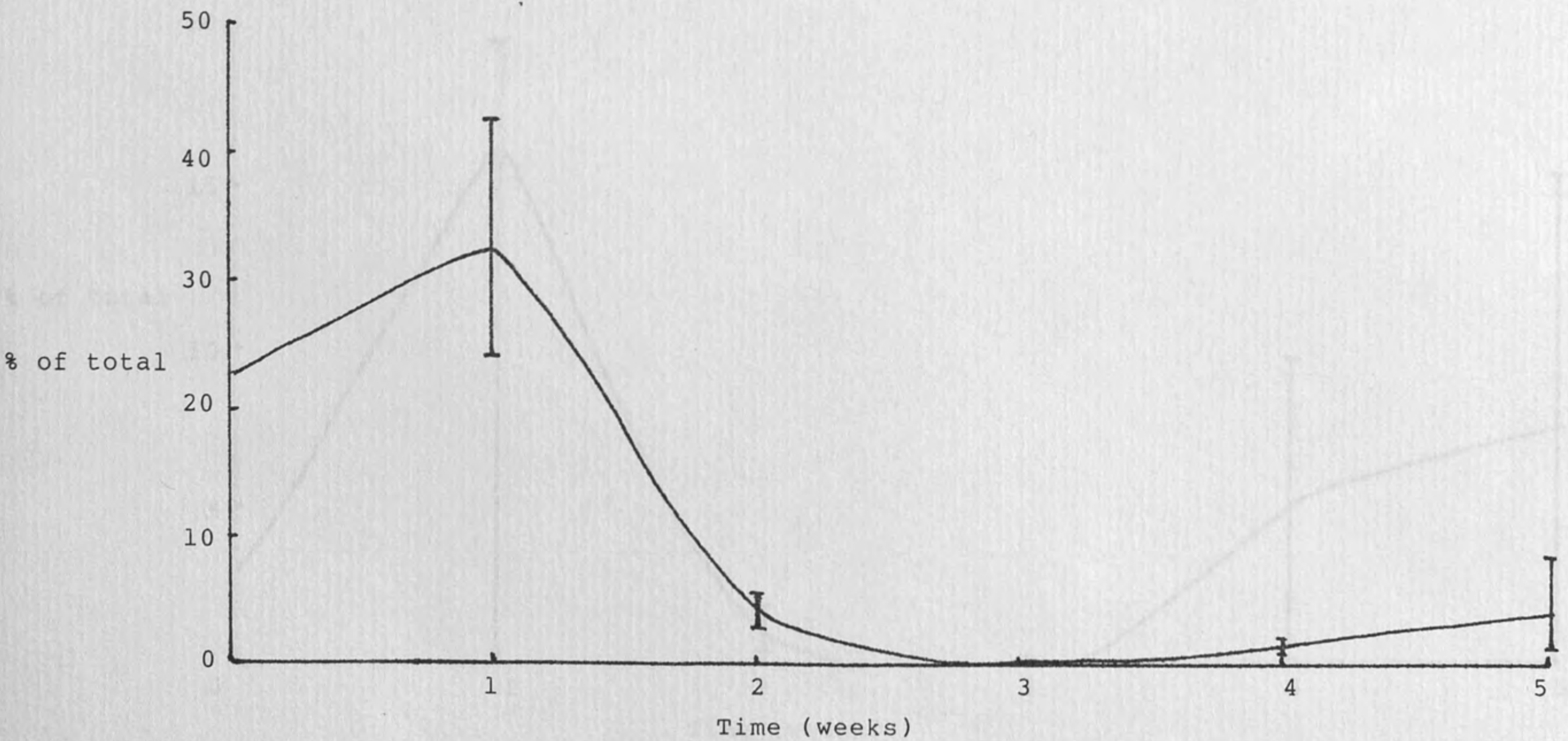


Figure 53: Change in number of _Klebsiella_, cultured on MacConkey agar as a percentage of the total number of colonies cultured on NA, colonising sterile alder leaves _in vitro_.

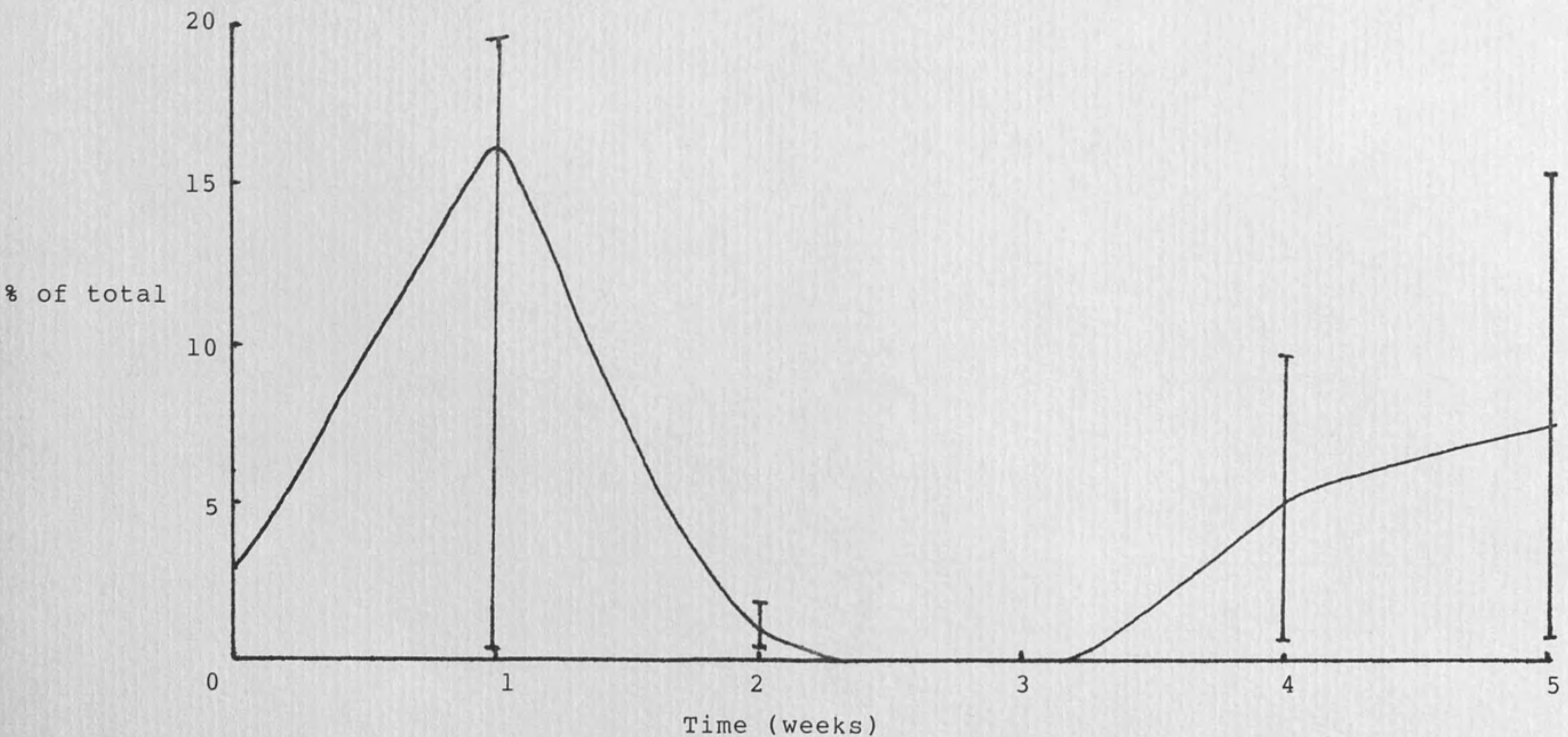
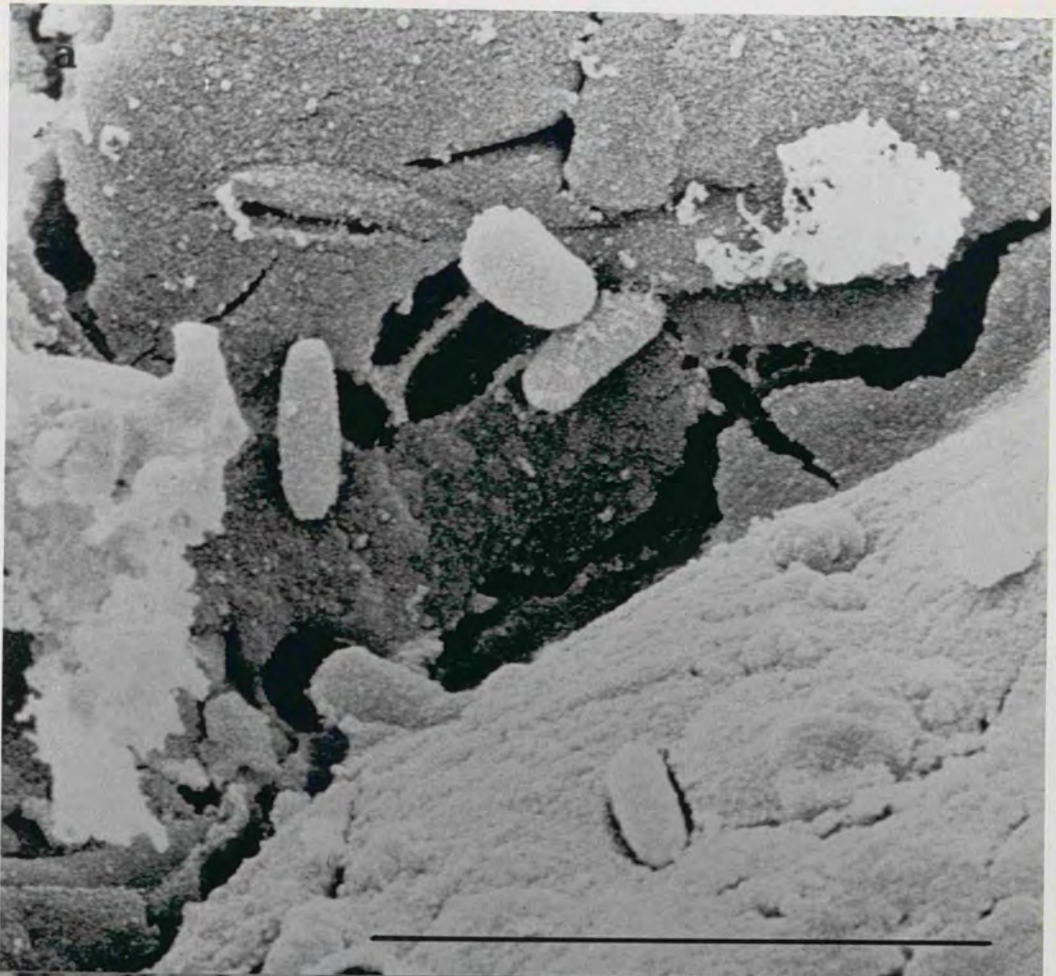


Figure 54: Change in number of _Aeromonas_, cultured on PAA as a percentage of the total number of colonies cultured on NA, colonising sterile alder leaf discs _in vitro_.

Figure 55: Burrow holes and erosion pits with associated microorganisms on alder leaves incubated *in vivo*. Figure 55a shows a group of three erosion pits possibly caused by the associated bacteria, and a rod-shaped bacterium in the process of burrowing. Figure 55b shows two uniformly-shaped round holes, likely to be burrow holes caused by bacteria. Bar measurements a=10µm, b=5µm.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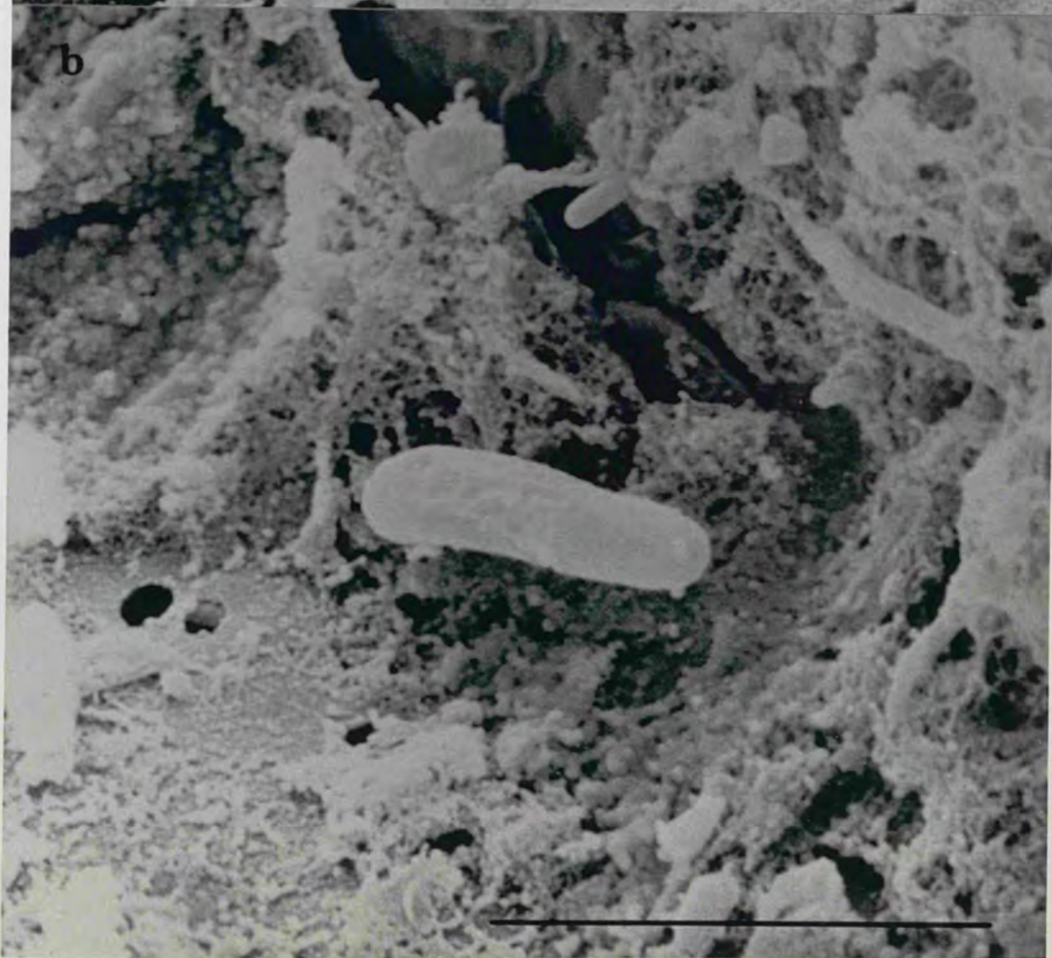
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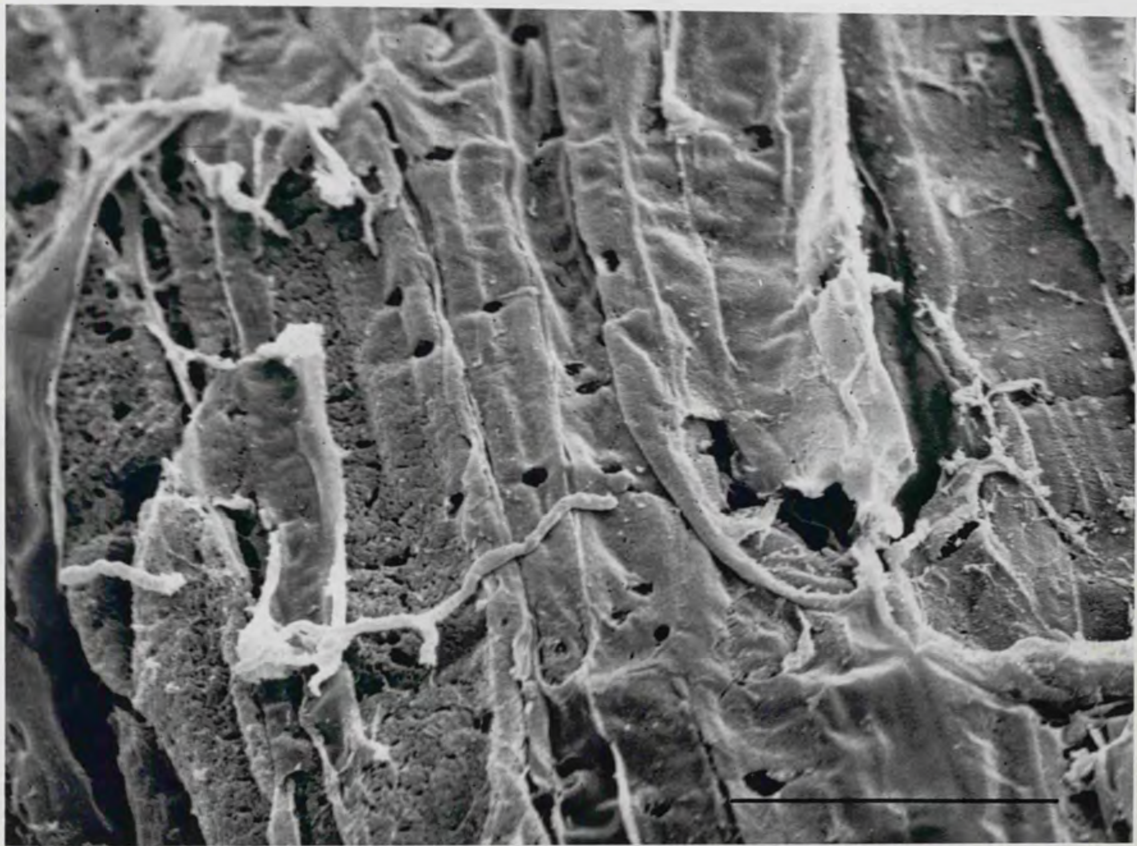
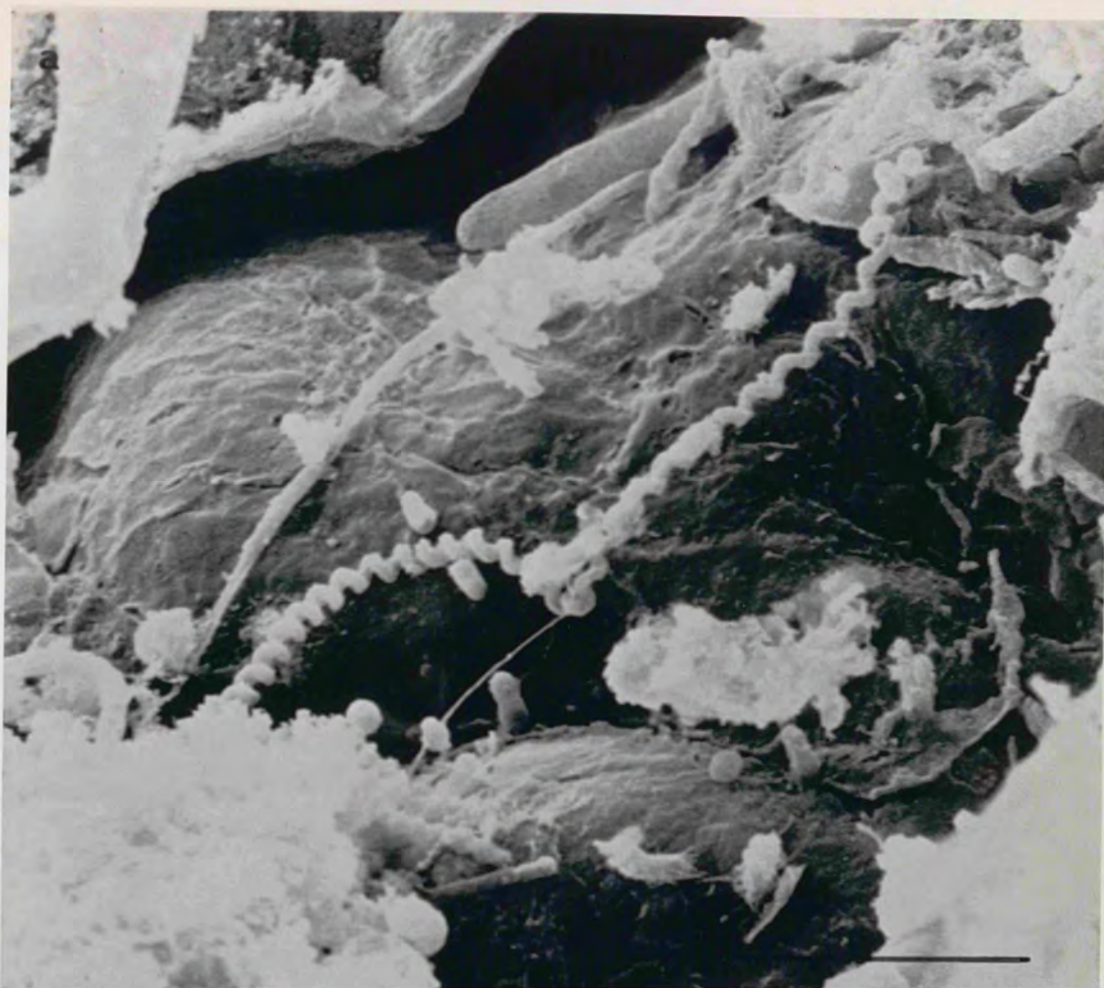
Figure 56: Burrow holes in sterile alder leaves incubated *in vitro* with a known mixture of microorganisms. Bar measurement= 20μm.



Figuree 57: Organism with spirochaete-like morphology observed on decaying alder leaves incubated *in vivo*. Bar measurements a=5μm, b=2μm.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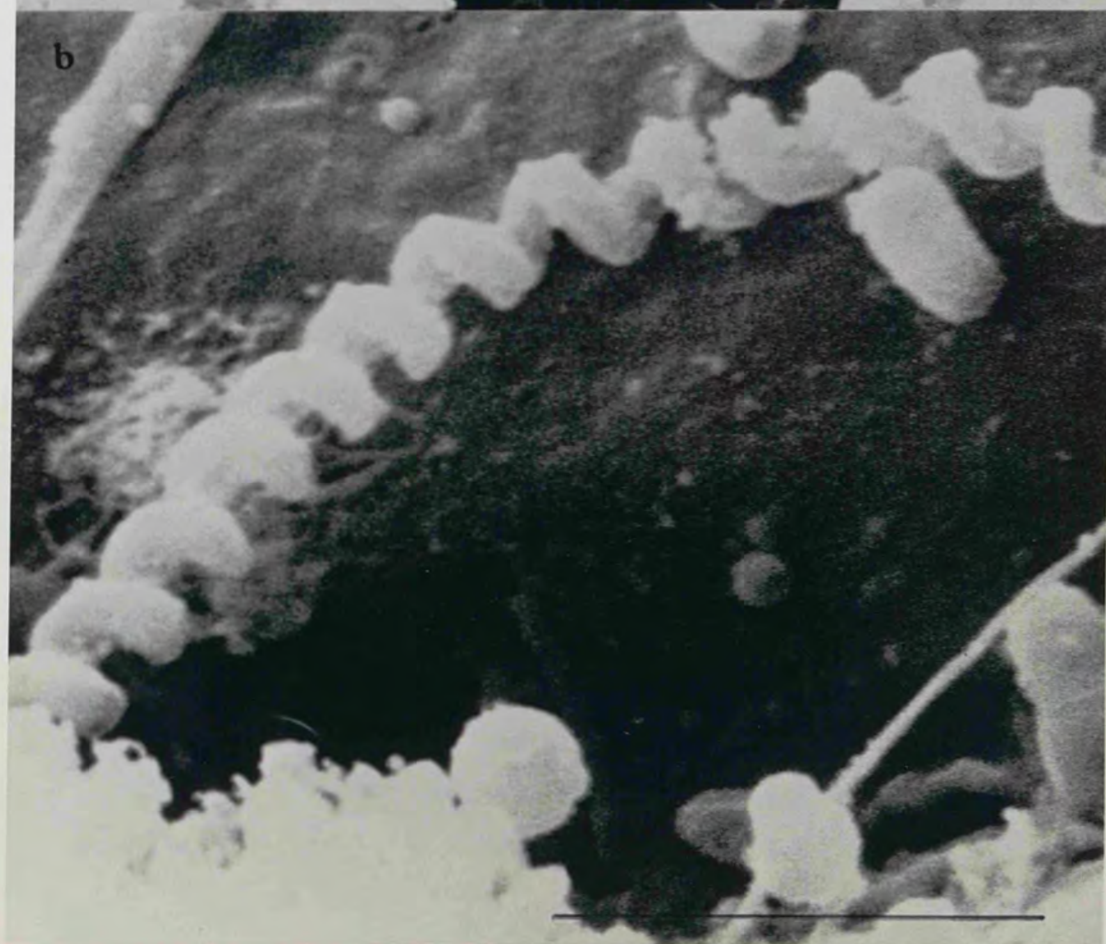
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Figure 58: Organism with spirochaete-like morphology which was highly motile observed on acridine orange stained preparations of alder leaves which had been incubated *in vivo*. Magnification 1000x.

The pectin, lignin, hemicellulose and cellulose conten
of dried alder leaves was calculated as shown in table
25.

The pectin, lignin, hemicellulose and cellulose content of dried alder leaves was calculated as shown in table 25.

Starting material and weight	Treatment	Polymer extracted	Final weight	% remaining	Polymer % (not corrected)
cell wall	hot water	pectin	7,05	70,5	29,5
cell wall (pectin extracted) 5g	acetic acid/ sodium chlorite extraction	lignin	4,73	94,6	5,4
cell wall (pectin and lignin) extracted 2,5 g	KOH extraction	hemi-cellulose A & B	1,84	66	26,4
Cellulose content calculated by difference:-					38,7

Table 25: Polymer content of dried alder leaf cell walls

i Pectinases

Isolates were tested for liquefaction of sodium polypectate media at different pHs in order to assess which organisms were more active in breakdown of this substrate. This was accomplished using growth and liquefaction of sodium polypectate gel as shown in table 26.

Isolate	pH 4,9	pH 7,0	pH 9,4
Xanthomonas	NG	+	+
Ps.cepacia/ chloraphis	-	+	+
Aeromonas	NG	-	-
Ps. maltophlia	NG	-	-
Alcaligenes	-	-	-
Ps.testosteroni	+	+	+
Ps.putida	-	-	-
Erwinia	+	+	+
Bacillus (spreading)	NG	-	-
Bacillus (discrete)	NG	-	+
Rhodococcus	NG	+	+
Ps.fluorescens	-	-	-
Cytophaga	NG	+	+
Proteus	-	-	-
Janthinobacterium	-	-	-

	pH		
Isolate	4,9	7,0	9,4
Streptomyces (grey)	-	-	-
Streptomyces (white)	-	-	-

Table 26: Growth and liquefaction of sodium polypectate gel by isolates

NG = no growth

Liquefaction of sodium polypectate gels showed that Rhodococcus, Erwinia, Ps. testosteroni, Xanthomonas and Cytophaga were active at neutral and alkaline pH and Bacillus at alkaline pH only. Few organisms grew at acid pH which suggests an intolerance. This is however not indicative of an inability to produce an enzyme active at low pH and therefore cup plate assays, which do not require the growth of an organism at sub-optimal conditions were undertaken. Enzymes were first induced in liquid media containing sodium polypectate as its sole carbon source. After 18 hours the supernatants were clarified by centrifugation and small aliquots added to precut w lls in sodium polypectate assay gels in petri dishes. The supernatants were allowed 24 hours to affect breakdown of the substrates and the plates were then developed using cetrimide which precipitates intact sodium polypectate rendering it opaque and leaving areas where substrate breakdown has occurred clear. Included in these results are the activities of two Cytophaga sp which were the generous gift of Dr D.E. Stead (MAFF., Harpenden, Herts.). Zones of breakdown in cup plate assays were produced at neutral pH by Bacillus, Ps. fluorescens, Ps. putida and Ps. cepacia/chloraphis and at alkaline pH by Erwinia, Xanthomonas and Cytophaga (table 27).

Isolate	pH 4,9	pH 7,0	pH 9,4
Xanthomonas	–	–	+
Ps.cepacia/ chloraphis	–	–	–
Aeromonas	–	–	–
Ps.maltophilia	–	–	–
Alcaligenes	–	–	–
Ps.testosteroni	–	–	–
Ps.putida	–	+	–
Erwinia	–	+	±
Bacillus (spreading)	–	–	–
Bacillus (discrete)	–	–	–
Rhodococcus	–	–	–
Ps.fluorescens	–	–	–
Cytophaga	–	–	+
Proteus	+	+	+
Janthinobacterium	–	–	–
Streptomyces (grey)	–	+	+
Streptomyces (white)	–	+	+

Isolate	pH 4,9	7,0	9,4
Cytophaga sp NCPPB 3090	+	+	+
Cytophaga A 3721	+	+	+

Table 27: Breakdown of sodium polypectacte in cup plate assays using supernatants from pectinase inductions

Results obtained from liquefaction of sodium polypectate gels and cup plate assays show that the majority of pectin degrading enzymes produced by isolates were active at neutral and alkaline pH. Enzymes were therefore induced in liquid media containing sodium polypectate or pectin in order that the supernatants could be more specifically assayed for the presence of these enzymes and that the activities of the isloates could be quantified and compared.

Pectate lyases are active at alkaline pH and activity was measured by monitoring the increase in absorbance at 235 nm as unsaturated uronide units are formed. Enzyme activity in 18 hour cultures was arbitrarily expressed as units/ml supernatant (Table 28). Included in these results are the activities of 4 cultures of known pectinase producers which were the kind gift of Dr D.E. Stead (MAFF, Harpenden, Herts).

Isolate	Pectate lyase activity/ml supernatant after 18h induction
Xanthomonas	2,76
Ps.cepacia/ chloraphis	none
Aeromonas	none
Ps.maltophilia	none
Alcaligenes	0,467
Ps.testosteroni	none
Ps.putida	0,457
Erwinia	none
Bacillus (spreading)	none
Bacillus (discrete)	none
Rhodococcus	none
Ps.fluorescens	0,91
Cytophaga	1,75
Proteus	none
Janthinobacterium	none
Streptomyces (grey)	none
Streptomyces (white)	none

Isolate	Pectate lyase units/ml supernatant after 18h induction
Cytophaga sp. NCPPB 3090	0,994
Cytophaga sp. A3721	1,33
Erwinia chrysanthemi NCPPB 2844	2,62

Table 28: Activity of pectate lyases induced by 18h culture of isolates in pectate lyase induction medium

Pectinmethyl esterases are active at neutral pH and act preferentially on methylated pectin. Enzyme activity is measured as decrease in pH which results from demethylation of the substrate. Pectinmethyl esterase activity was not detected in any of the culture supernatants from isolates or from the reference cultures noted in table 28.

ii Xylanases

The ability of isolates to affect breakdown of xylan was tested using production of a clearing surrounding growth on xylanase assay medium (table 29).

Organism	Xylan Breakdown
Xanthomonas	-
Ps.cepacia/chloraphis	-
Aeromonas	+
Ps.maltophilia	-
Alcaligenes	-
Ps.testosteroni	-
Ps.putida	-
Erwinia	-
Bacillus (spreading)	+
Bacillus (discrete)	+
Rhodococcus	-
Ps.fluorescens	-
Cytophaga	-
Proteus	-
Janthinobacterium	-
Streptomyces (grey)	+
Streptomyces (white)	+

Table 29: Breakdown of xylan in xylanase assay plates by isolates

iii Cellulases

Three different sources of cellulose were used to induce production of cellulases. Cultures were grown in mineral medium containing either solka floc or RBB dyed cellophane or in a mineral medium which contained dyed cellophane and cellobiose as an additional inducer. Cellulases were assayed

for by reducing sugar analysis of reaction products formed by incubation of supernatants from solka floc cultures and CMC and also by visual assessment of release of RBB from cellophane.

Positive results were recorded only for the Streptomyces spp (table 31).

Isolate	Release of RBB from cellophane		Cellulase units
	with cellulose	no cellulose	per ml
Streptomyces (grey)	+	+	6,2
Streptomyces (white)	+	+	5,2

Table 30: Action of Streptomyces spp on RBB dyed cellophane, assayed visually and on CMC, after induction with solka floc, assayed by measurement of reducing sugars.

7. Loss in dry weight of alder leaves as a result of microbial activity

Pure cultures of bacteria and of the aquatic Hyphomycete *Tricladium splendens* and a mixed culture of the bacteria and aquatic Hyphomycete were inoculated into mineral medium containing 1 g each of sterile alder leaves and incubated with shaking. An uninoculated control flask containing 0,2% sodium azide was also incubated. The dry weights of the leaves were assessed after 24 days incubation by filtration of the contents of each flask, drying to constant weight at 40°C and weighing to the second decimal place. The loss in dry weight of leaves incubated with microorganisms was compared with the control using a one way analysis of variance and Turkey's Honest Significant Difference (Snedecor 1956).

Thus, there were 21 treatments with 3 replicates of data each (table 4, Appendix).

$\Sigma\Sigma = 40,762.$

$T = \Sigma x^2 = 27,401$

$T_0 = {}^{\Sigma\Sigma}/K = \quad {}^{40,76}/63 = 0,647$

$T_T = \Sigma x^2/n-1 \quad {}^{81,39}/3 = 27,13$

$SS_{treatment} = 27,13 - 0,647 = 26,48$

SS_{total} = 27,401 - 0,647 = 26,75

Source	df	s.s.	m.s.	F
Treatments	20	26,48	1,324	207
Error	42	0,27	0,0064	
Total	62	26,75		

From f tables F_{20} + 40 0,05 = 1,84

F_{20} + 40 0,01 = 2,37

F_{20} + 40 0,001 = 3,40

Turkey's Honest Significant Difference

$HSD = S\bar{x} \times Q$

Q from table with 42 df and 21 treatments = 5,36

$$S\bar{x} = \sqrt{\frac{EMS}{\text{no. in each treatment}}}$$

$$= \sqrt{\frac{0,0064}{3}}$$

$$= 0,046$$

$$HSD = 0,046 \times 5,36 = 0,246$$

Thus, in order to calculate whether a significant reduction in dry weight of the leaf has been affected by the activity of the microorgansims, the mean value, x is subtracted from x control and if $x_{control} - \bar{x}_{sample} > 0,246$ then $\bar{x}_{control} \neq x_{sample}$ and the null hypothesis is rejected.

Isolate numbers

	control	225	226	15	179	210	197	163	166	174
Σx	2,972	1,37	1,96	2,14	1,73	1,82	2,10	1,99	1,87	2,24
$\bar{x}$	0,99	0,46	0,65	0,71	0,58	0,61	0,70	0,66	0,62	0,75

	201	149	168	169	174	155	219	222	193	T. splendens	Bacteria and T. splendens
Σx	1,84	1,97	2,17	2,04	2,24	1,80	1,64	1,57	1,96	1,89	1,45
$\bar{x}$	0,61	0,66	0,72	0,68	0,75	0,60	0,55	0,52	0,65	0,63	0,48

Table 31a: Sums of dry weights of leaves and mean values thereof for use in one way analysis of variance

Isolate	$\bar{x}$ control - $\bar{x}$ sample for dry weights of alder leaves after incubation with microorganisms	Significance as calculated by Turkey's HSD
Xanthomonas	0,34	+
Ps. cepacia/ chloraphis	0,41	+
Aeromonas	0,25	-
Ps. maltophilia	0,38	+
Alcaligenes	0,27	+
Ps. testosteroni	0,29	+
Ps. putida	0,37	+
Erwinia	0,31	+
Bacillus (spreading)	0,38	+
Bacillus (discrete)	0,33	+
Rhodococcus	0,39	+
Ps. fluorescens	0,33	+
Cytophaga	0,47	+
Proteus	0,44	+
Janthinobacterium	0,28	+
Streptomyces (grey)	0,53	+
Streptomyces (white)	0,34	+
Tricladium splendens	0,36	+
Bacteria and T. splendens	0,51	+

Table 31b: Differences between mean dry weights of alder leaves from an uninoculated control and from leaves incubated with microorganisms after 24 days and significance of difference as calculated by one way analysis of variance and Turkey's HSD.

DISCUSSION

Investigation of microorganisms and their roles in the environment is a complex issue because of the many varying influences and results are often dependent upon the techniques employed. Brock (1987) favoured the use of systems modelling, direct microscopy and microscopic activity studies, biomass/activity studies and integrated field and laboratory systems for the study of microbial ecology. He considered the merits of each type of study and a summary of his appraisals is given below.

Brock considered that systems modelling made a realistic and mathematical approach to the study of microbial ecology and although pure cultures are often used it is done in such a way that the natural environment is mimicked. Experimental conditions are also controlled so that they approximate the natural conditions ie low nutrient concentrations, low bacterial density and slow growth rate.

Direct microscopic examination is favoured because it allows observation of a microorganism in its natural habitat. This is considered important because many of the factors controlling the microenvironment are not reflected at the macroenvironmental level and therefore cannot be measured by conventional methods. The use of microscopic microautoradiography can also be used in activity studies, thus further maintaining the organism in its natural environment.

Quantifying microbial biomass by measurement of microbial activity was another approach to microbial ecology favoured by Brock although he cautions that in studies of this nature the conditions of the microenvironment are all too often ignored. He also favoured integration of field and laboratory studies although he considered that their application was generally limited to fairly well-defined habitats such as lakes and estuaries.

Brock considered that pure and mixed culture studies, viable counting and species diversity were inadequate methods for the study of microbial ecology. His reasons summarized, were that pure cultures dealt with under highly artificial conditions in rich media were not model systems which mimic nature, but merely physiological studies, particularly those which employ the use of chemostats or enrichment cultures which, after a number of successive transfers or period of enrichment contain only a limited number of organisms in an environment which is nothing like the natural environment. Brock also considered viable counting inadequate since it does not produce a realistic community. Viable counting selects for unicellular organisms and organisms which spread and also encourages growth of dormant cells, but selects against organisms which cannot grow well against the resistance offered by the solid substrate. Species diversity was considered to derive a diversity index which relates merely to those organisms isolated and not necessarily those in the natural environment.

The bacterial breakdown of allochthonous litter in freshwater has received little attention and in general the more detailed studies have concentrated on the role played by aquatic Hyphomycetes. Bacterial populations were estimated but the contribution made to the degradation process was not considered. Similarly in studies of wood degradation the role of fungi was considered and Holt et. al. (1979) considered that the effect of bacteria on wood decay had not been fully investigated because (a) of the belief that they are unimportant in wood decay, (b) limitations imposed by techniques and available instrumentation, (c) lack of interest, and (d) difficulties in characterising non-clinical isolates.

The objective of this study was therefore to address the presence and role played by bacteria on degrading alder leaves in a freshwater stream. Use of only one leaf species allowed for a more in-depth study to be made and evidence suggests that bacteria which colonise degrading leaves in rivers are river and runoff associated (Suberkropp and Klug, 1976) and the range of organisms available to colonise allochthonous litter is therefore limited and influenced by the river. The chemical composition of the leaf is known to influence rate of decompostion but the mix of colonising organisms is generally similar on different leaf species incubated at the same location (Triska, 1970; Suberkropp and Klug, 1976; Chamier, 1980).

Bacterial colonisation of alder leaves was monitored *in vivo* by SEM and fluorescence microscopy. High magnifications

used in SEM allowed accurate determination of bacterial morphology, orientation of individual cells with respect to one another and to the leaf structures and also their association with polysaccharide. Enumeration of organisms under fluorescence microscopy assisted in verifying perceived increases in numbers of bacteria colonising alder leaves observed under SEM.

A succession of bacterial morphotypes on degrading alder leaves was demonstrated using SEM. It is likely that the ordered nature of the colonisation was in response to a variable related to the substrate itself or else the products of metabolism of other organisms already present on the leaf.

SEM showed that colonising organisms were closely associated with the leaf surfaces and not the debris which accumulated on the leaf. Bacteria were never observed attached to diatoms and rarely attached to fungal hyphae. This suggests that the association of bacteria with degrading alder leaves was as a result of the influence of leaf and not the other associated microflora or its byproducts. A survey of the enzymes produced by isolates from degrading leaves and also of their ability, in pure and mixed cultures, to affect a loss in any weight of alder leaves has shown that the isolates do produce enzymes capable of breakdown of cell wall structural polymers and that they also affect a reduction in dry weight of leaves *in vitro*. This evidence suggests that the ordered pattern of colonisation of alder

leaves by bacteria was due at least partly to availability of polysaccharides in the leaf.

Similar patterns of colonisation of alder leaves were observed on samples incubated in the river during periods when temperature was decreasing or increasing. One experimental run was also conducted during summer when the average water temperature during the degradation period was 17,7°C and although sampling was too infrequent to discern a colonisation pattern, the mixture of morphotypes observed on these samples was the same as that observed in the experimental runs where mean temperatures ranged from 7,3°C to 13,4°C. This suggests that most of the organisms observed on these leaves were facultative psychrophiles as defined by Frobisher et. al. (1974) whose definition was that the organism should be capable of growth at 5°C or lower but whose growth temperatue optimum is 25-35°C. 65% of the isolates selected for further work in this study grew at 4°C thus supporting this observation. This evidence supports Suberkropp and Klug's (1976) findings that their bacterial isolates from oak and pignut hickory leaves were able to grow at low temperature but displayed temperature optima of approximately 26°C. Bell et. al. (1980) also found that the bacterial populations of two Canadian rivers were able to grow at temperatures ranging from 4°C to 27°C and higher.

The attachment of bacteria to surfaces is a common phenomenon (Zobell, 1943; Marshall et. al., 1971; Fletcher and Floodgate, 1973; Costerton et. al., 1978; Allison and

Sutherland, 1987) and a key feature of a colonisation pattern is that microorganisms become attached or closely associated with the substrate. Cocco-bacilli and occasionally intermediate length bacilli were observed on alder leaves in association with polysaccharide 48 to 72 hours after commencement of colonisation and for 3 weeks thereafter. It is not known whether polysaccharide became indistinguishable from debris after 3 weeks incubation or whether polysaccharide production had ceased.

Bacterial attachment to surfaces is mediated by polysaccharide (Jones et. al., 1969; Geesey, 1982) or structures such as fimbriae (Duguid, 1959; Opperman and Wolfson, 1961; Rosenberg et. al., 1982), pili (Weiss, 1973) and holdfasts (Zobell, 1943; Fletcher and Floodgate, 1973). Polysaccharide association, in addition to being a vehicle for attachment, also offers a number of advantages to the microorganism. Polysaccharide fibres can function as a food reservoir since nutrients, which are positively charged, would bind to the negatively charged fibres (Costerton et. al., 1978; Geesey, 1982); a polysaccharide covering or capsule also affords protection against amoebic attack, bacteriophage endotoxins and aggressins (Wilkinson, 1958).

Marshall et. al., (1971) showed that the initial binding of a microorganism to a surface is characterised by being a loose, reversible association and positive and negative forces are thought to counteract one another thus initiating a state of equilibrium. This reaction is non-specific. Formation of a permanent, irreversible bond is associated

with polysaccharide production (Zobell, 1943; Marshall et. al., 1971; Fletcher and Floodgate, 1973; Cagle, 1975). Allison and Sutherland (1987) however postulated that the mechanism of initial attachment may be a specific interaction between a cell surface receptor and the substrate and that the polysaccharide production functions in development of microcolonies or the biofilm, but not in attachment per sé.

The exclusive association of only two morphotypes with polysaccharide on alder leaves tends to suggest that it was produced by these organisms and not by any other morphotypes observed. SEM observation of pure cultures of some organisms used for further work in this study also demonstrated that some isolates did not produce polysaccharide while others were never observed in association with polysaccharide. This suggests that the attachment of apparently non-polysaccharide producing organisms is mediated by other factors. Some isolates which were negatively stained for TEM viewing of flagella also possessed fimbriae which are mediators of attachment (Duguid, 1959; Opperman and Wolfson, 1961; Rosenberg et. al., 1982).

The delay between colonisation of alder leaves in the stream and the appearance of polysaccharide could be for any one of three possible reasons: this could be the period between reversible and irreversible binding (Zobell, 1943); this could be the length of time required by organisms to reach stationary phase of growth when polysaccharide production

becomes more prevalent (Costerton et. al., 1978); or that during the early stages of colonisation the organisms produce polysaccharide but in quantities too small for observation under SEM.

Polysaccharide-associated bacteria have been observed on timber blocks (Holt et. al., 1979) and on delignified timber (Holt and Jones, 1983) incubated in microaerophilic and anaerobic marine and freshwater muds. Federle and Vestal (1982) also observed "bacteria-like" forms enmeshed in slime on the surfaces of Carex leaf litter incubated in a freshwater lake. This slime became less evident as incubation proceeded and was no longer in evidence after 28 days incubation.

Organisms possessing holdfasts were frequently observed on the surfaces of alder leaves. This feature, pili and polysaccharide production serve to demonstrate the variety of modes of attachment used by bacteria in association with degrading leaf litter and thus the versatility of this flora.

Brock (1987) has commented that certain parameters in the microenvironment are not always reflected in the macroenvironment. The presence of certain microorganisms within a microenvironment however offers a system whereby predictions can be made from their presence. Prosthecate (stalked) bacteria and branched actinomycete-like filaments were freqeently observed on decaying alder leaves and Chamier (1980) has demonstrated that aquatic Hyphomycetes

are also always present. This is indicative of a highly aerobic environment. Their presence suggests that oxygen levels throughout the leaf pack were high. This is probably because the leaf packs were small (5 leaves ≈1 g) and the river fast-flowing (1m/5 secs, Chamier, 1980) and shallow. The accumulated debris and biomass on alder leaf was never observed to exceed approximately 50 µm and Ladd et. al (1979) showed that a layer of microbial biomass or debris of at least 70 µm was required in epilithic microbial populations before oxygen became limited and anaerobic conditions would probably arise in the lower levels of the biofilm.

Organisms of spirochaete morphology were also observed on decaying alder leaves although their presence was generally not expected. Spirochaetes are more commonly associated with highly anaerobic environments and were observed by Holt and Jones (1983) in anaerobic muds. The organisms observed in this study under fluorescence microscopy were highly motile and therefore unaffected by exposure to oxygen. This tends to suggest that they were either aerobic (Leptospira sp) or facultatively anaerobic (Spirochaeta sp). The presence of a terminal hook, observed on all helicaly coiled organisms observed under fluorescence microscopy, is indicative of Leptospira sp which is indeed a free-living aerobic spirochaete (Holt, 1977). These organisms were not attached to the leaf surface as evidenced by their motility and most of them would therefore have been washed off the leaf surface during SEM preparation and therefore

infrequently observed under SEM. In view of the motile nature of these organisms it would seem unlikely that they contributed greatly to leaf degradation. Their association with degrading leaves is likely to be as scavengers of the products of the metabolism of other associated flora.

Many of the isolates obtained from alder leaves were motile when tested in pure culture. It cannot, however, be concluded that they were also motile under the conditions on the leaf and thus unimportant and indeed SEM of sterile alder leaves incubated with pure cultures of isolates has shown that all isolates tested colonised the leaf discs regardless of absence or presence of motility.

SEM observations showed the presence of erosion pits in alder leaves and also some burrowing organisms. The appearance of holes in alder leaves created by bacteria in field samples did not appear to follow a colonisation pattern although during the later stages of decay discernment of a colonisation pattern was not possible because the leaves became encrusted with layers of debris. This debris would also have masked burrow holes.

Burrow hole formation in the <u>in vitro</u> samples, however, occurred after 3 weeks incubation. Bacteria were never observed in association with the holes, but since the leaf discs were sterilised before used and burrow holes were not observed for the first three weeks of incubation, the holes must have resulted either from bacterial action or physical abrasion. The latter explanation appears unlikely in view

of the uniformity of the holes. The diameters of approximately 1,3 μm also suggest that the holes were created by bacteria.

All bacteria observed burrowing into in vivo samples were rod-shaped. The identities of those burrowing and erosion trough bacteria is unknown, but it is significant that burrow holes were observed in the in vitro samples which were incubated with a known mixture of bacteria consisting of Bacillus, Pseudomonas, Aeromonas, Klebsiella, Alcaligenes, Rhodococcus, Xanthomonas, Streptomyces and Erwinia. The occurrence and ability of bacteria to burrow into wood and also to form troughs and pits has been demonstrated on wood samples by Holt and Jones (1978; 1979; 1983) and Mouzouras et. al. (1986). Mouzouras et. al. (1986) also demonstrated that all burrowing organisms they observed were rod-shaped and their findings are supported by this work.

Enumeration by fluorescence microscopy of organisms colonising degrading alder leaves in 2 experimental runs (2 and 5) showed that colonisation commenced within 24 hours of incubation in the river, and that the number of microorganisms did not change significantly during the following seven days of incubation. Correctly scaled graphs of these data show gradual (but statistically insignificant) increases in bacterial number which could account for the perceived increased in numbers of microorganisms observed under SEM. This was due to both proliferation of colonised organisms and initial colonisation by other organisms. A

rapid initial colonisation of fallen leaves is expected to occur in response to leaching of soluble low molecular weight compounds from the leaves, since these compounds are easily assimilable by bacteria. Although these results do not show a rapid initial colonisation of leaves per sè, organisms were not enumerated until the leaves had been incubated for 24 hours and the leaves were essentially devoid of bacteria prior to incubation in the river (SEM and fluorescence microscopy), so the numbers of bacteria present after 24 hours must therefore be considered to be a significant increase from an uncolonised leaf.

The numbers of colonising organisms increased significantly after 7 days incubation during run number 5, but were significantly reduced in run number 2, both eventually reaching a plateau at which no significant changes in bacterial numbers occurred and which persisted until the end of that decay period. There was no apparent reason for the increase in numbers of bacteria in one run, but decrease in the other after 7 days incubation. The consistency of these results would however suggest that those observations were not due to possible counting errors. It is likely that localized leaf pack characteristics and differences in water flow rate accounted for these differences. This is supported by comparison of these results with those obtained by enumeration of organisms colonising sterile alder leaf discs <u>in vitro</u> which showed a significant decrease in bacterial numbers after 7 days incubation reflecting the observations made during experimental run number 2. The

consistency of an <u>in vitro</u> environment would not reflect physical changes in an environment <u>in vivo</u> and the results would therefore be expected to approximate the conditions in an environment which experienced the least change, in this case that of experimental run number 2. The implication is therefore that environmental changes occurred during the course of run number 5 which displayed an increase in bacterial number and that under regulated conditions a decrease might be expected.

Both experimental runs and the <u>in vitro</u> run showed that the numbers of bacteria reached a plateau towards the latter half of the degradation process, from which point on no significant changes in bacterial numbers were observed. This supports SEM observations that by half way through leaf degradation representatives of all morphotypes were observed and the leaf was thus "fully colonised".

The <u>in vitro</u> colonisation of alder leaf discs reflects that observed during experimental run number 2 more closely than run number 5. The one difference between <u>in vitro</u> colonisation and run no 2 is that after 24 hours of incubation there was a significant reduction in bacterial numbers in the <u>in vitro</u> run. This was followed over the next 4 days by a plateau during which time no significant change occurred and the numbers of organisms recorded on day 4 and 7 did not differ significantly from the number of bacteria on leaf discs incubated for 24 hours. It is thus likely that the observed reduction in numbers might be due

to counting errors or conditions peculiar to *in vitro* conditions.

These results show that colonisation commences within 24 hours of incubation of leaves either *in vitro* or *in vivo*, that bacterial numbers remain steady within the first 7 days of incubation regardless of length of time required for alder leaves to be degraded and that towards the latter half of the decay period a steady state in terms of bacterial number is reached. These periods could be described for alder leaves as an initial colonisation period lasting 24 hours, an establishment period lasting 7 days, an instability period which accounts for one third to half the length of time required for leaf degradation to occur and a stabilisation period which last for approximately 3 weeks.

The actual numbers of bacteria in these runs are not strictly comparable because consistent magnification was not used in all experimental runs and while within one run they are comparable there are differences between results obtained using different magnifications which have been discussed in section 2 in Results.

SEM observation of some of the isolates selected for further investigation in this study had shown that at least two possessed morphologies similar to those observed *in vivo* and similarly that some cocco-bacilli produced polysaccharide. Colonisation of sterile alder leaves and leaf discs *in vitro* by a mixture of known genera, although limited by not being the full complement of bacterial morphotypes bore

similarities to the colonisation pattern obvserved in vivo. Colonisation commenced within 24 hours and was initiated by cocco-bacilli which colonised in isolation or in small groups.

Cocco-bacilli were frequently observed in association with polysaccharide. Intermediate length bacilli colonised after cocco-bacilli and although they never appeared to be more abundant than cocco-bacilli showed a steady increase in numbers and size of group. This morphotype was also associated with small amounts of polysaccharide. Branched hyphae probably Streptomyces spp colonised the leaves in vitro after the cocco-bacilli and intermediate length bacilli had become established. Branched organisms were, however, poorly represented in in vivo samples. This could have been because they were not abundant, or that they were abundant but indistinguishable from the accumulation of debris on the later samples, or that the mycelial growth was within the leaf. Spores produced by such a mycelium would be most likely to be produced on the surface of the leaf so that they could be easily dispersed. It is unlikely that the delicate streptomycete spore-chain would remain intact and identifable as a spore-chain for any time in a water current and it not therefore expected that spore chains would be observed. Suberkropp and Klug (1974) reported similarly that they rarely observed aquatic Hyphomycete mycelium on oak and hickory leaves under SEM, but that they frequently observed the spores. Aquatic Hyphomycete spores are easily distinguishable by their tetraradiate spores

(Ingold, 1975). Suberkropp and Klug suggested that the mycelial growth of the aquatic Hyphomycetes was present within the leaf. An organism capable of gaining access to, and growing within a substrate not yet attacked by other microorganisms would be at a competitive advantage. If this hypothesis is correct then it could be that increased competition form the microflora and fauna in the _in vivo_ situation would induce _Streptomyces_ spp to produce mycelia within the substrate and the simpler and less competitive environment in the _in vitro_ system would not, hence the observation of a well developed surface mycelium.

On both _in vitro_ and _in vivo_ samples the numbers of any one particular morphotype tended to increase once they had become established and then to persist through the duration of the experimental run or decay period, usually slightly less abundantly. The ordered colonisation pattern observed in _in vitro_ samples which had been inoculated with similar quantities of each organism suggests that colonisation was in response to a variation in the substrate. A random colonisation pattern would have been observed if this were not the case. Colonisation and proliferation of these organisms on alder leaves, which were the only _in vitro_ nutrient source available, also demonstrates that these organisms were able to utilize the leaves as a nutrient source.

The similarity between colonisation of alder leaves _in vivo_ and in this simple _in vitro_ system also suggests that the

isolates obtained were a representative mixture of the bacteria present on degrading alder leaves *in vivo*.

Enumeration of colonised organisms from *in vitro* samples showed no consistent pattern of colonisation by the genera inoculated and these results could therefore not be used as comparison. The work was labour intensive, even at an exploratory level and if any meaningful result could have been achieved this work would have had to be replicated many times. It was therefore considered inappropriate for one worker to undertake such an exercise.

Many studies, including this one, have demonstrated the presence of bacteria on degrading leaf litter in freshwater streams. Few attempts have however been made to characterise the bacteria present on these leaves. Suberkropp and Klug (1976) characterised 12 different genera associated with stream water and oak and hickory leaves. These were *Flavobacterium*, *Flexibacter*, *Pseudomonas*, *Acinetobacter*, *Achromobacter*, *Chromobacterium*, *Serratia*, *Alcaligenes*, *Bacillus*, *Cytophaga*, *Sporocytophaga* and *Arthrobacter*. Thirteen different bacterial genera were isolated from decaying alder leaves in this study. These were *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Erwinia*, *Aeromonas*, *Cytophaga*, *Rhodococcus*, *Proteus*, *Streptomyces*, *Janthinobacterium*, *Xanthomonas*, *Escherichia* and *Klebsiella*.

Some similarities exist between organisms isolated in both studies. *Pseudomonas*, *Bacillus*, *Alcaligenes*, and *Cytophaga* were isolated in both studies as well as a number of enteric

bacteria. Pseudomonas, Bacillus and Alcaligenes are nutritionally versatile organisms and their occurrence is therefore not unexpected. Bacillus is also well adapted to survival of adverse conditions by virtue of its ability to sporulate. Pseudomonas and Bacillus have been reported to produce pectin and cellulose degrading enzymes (Enari, 1983; Zucker and Hankin, 1970; Nagel and Vaughn, 1961) and some species are also plant pathogens (Stead in press 1987a, Lelliot et. al., 1966).

Flavobacterium was not isolated in the present study but was found on oak and hickory leaves. However, Cytophaga was isolated in both studies and this genus is very closely related to Flavobacterium. Furthermore, classification guidelines for these genera have been revised in Bergey's Manual of Systematic Bacteriology (Krieg et. al., 1984) and members of the genus Flavobacterium have been further restricted to those organisms with low G & C content. Flavobacterium pectinovorum was consequently reclassified as Cytophaga johnsonae. The type culture F. aquatile was also considered to be more closely related to Cytophaga. The two genera are listed as being very closely related. It is thus likely that the Flavobacterium and Cytophaga isolated by Suberkropp and Klug share many characteristics with the Cytophaga spp isolated from alder leaves. Some Cytophaga isolates from alder leaves might also have been classified as Flavobacterium using the same procedures as Suberkropp and Klug.

Cytophaga sp are indicative of a woodland environment and they are well suited to this environment because they elaborate enzymes which attack complex plant cell wall polysaccharides such as cellulose and lignin (Bell et. al., 1980). They are also known plant pathogens and produce arrays of pectin degrading enzymes (Fogarty and Kelly, 1983; Stead in press, 1987a). Evidence also suggests that many species are psychrophilic and this would be a distinct advantage in a temperate stream (Bell et. al., 1980). Further similarities between the isolates from the two studies are not immediately apparent because Suberkropp and Klug's isolates were characterised according to criteria in Bergey's Manual of Determinative Bacteriology 7th Edition (Breed et. al., 1957) and those in this study according to the 9th edition of this text which was updated. Some genera and species listed in the 7th edition of the text were reclassified in the 8th or 9th editions and new genera have also been listed.

Janthinobacterium was isolated from degrading alder leaves. Members of the genus Chromobacterium, which is very closely related to Janthinobacterium, were isolated from oak and hickory leaves. Some Chromobacterium species were reclassified as Janthinobacterium in the updated versions of Bergey's Manual of Systematic Bacteriology (Krieg et. al., 1984) and the possibility also therefore arises that the Janthinobacterium isolates from alder leaves might have been classified as Chromobacterium using Suberkropp and Klug's methods.

Achromobacter and Alcaligenes, which are closely related were isolated by Suberkropp and Klug. Alcaligenes was also isolated from decaying alder leaves. The genus Achromobacter was listed in Bergey's Manual of Determinative Bacteriology 7th edition (Breed et. al., 1957), but not in the 8th edition because its members had been grouped together with the genus Alcaligenes. The genus was however revived and is listed in the 9th edition of this text.

In addition to those genera already mentioned five further genera were also isolated from degrading alder leaves. these were Erwinia, Xanthomonas, Aeromonas, Rhodococcus and Streptomyces. Xanthomonas and Erwinia sp are generally associated with plants either saprophytically, as pathogens or as constituents of the epiphytic flora (Krieg et. al., 1984) and it is therefore likely that these genera could be present on the leaf surface at the time of senescence. Isolation of these genera from degrading leaves in the river, however, is also indicative of their ability to survive in water.

Both genera possess phytopathogenic members (Stead in press, 1987a; Cuppels and Kelman, 1974, Miller and Schroth, 1972; Sands et. al., 1970) and are known producers of pectin degrading enzymes (Van Gijsegem, 1986; Fogarty and Kelly, 1983). Erwinia chrysanthemi has also been reported to produce endocellulases (Andro et. al., 1984).

Erwinias isolated from decaying alder leaves were classified as Erwinia herbicolor (synonymous with Enterobacter

agglomerans) and Enterobacter agglomerans by fatty acid profiling and API 20NE strips respectively. E. herbicolor, in addition to its association with plants, is also found in association with man, animals, soil, water and air and is known as Enterobacter agglomerans when isolated from clinical sources (Krieg et. al., 1984). The organisms isolated in this study have been referred to as Erwinia herbicolor because they are of non-clinical origin.

Aeromonas isolates were also obtained from degrading alder leaves. This genus is regularly isolated from freshwaters and sewage (Krieg et. al., 1984) and most strains grow at 5°C with optimum growth temperatures of 20 to 30°C. The organisms are thus psychrophilic and this supports SEM results which suggested that the colonising morphotypes were of a psychrophilic nature. Aeromonas liquefaciens is known to cause softening of plant material by production of polygalacturonic acid trans-eliminase (Hsu and Vaughn, 1969). The Aeromonas isolates in this study were, however, classified by both API 20NE and fatty acid profiling as A. hydrophila which is not a known pectinase producer. Nevertheless the presence of this genus in the river and also on degrading alder leaves is consistent with the distinguishing characteristics for this genus.

The occurrence of Aeromonas, Pseudomonas, Escherichia and Klebsiella, which are all common inhabitants of sewage, suggests that the River Bourne receives a certain amount of faecal contamination. This could be via seepage of domestic sewage, animal faeces or both. These findings support the

results obtained from Chamier's (1980) investigation of the physico-chemical status of the River Bourne which showed it to be mildy eutrophic.

Two actinomycetes, Rhodococcus and Streptomyces were isolated from decaying alder leaves. Actinomycetes are widely distributed in freshwater (Willoughby, 1969, 1966) but evidence suggests that they are mostly not part of the indigenous flora, but runoff associated and that they become active in such habitats only in the presence of a suitable substrate (Goodfellow and Williams, 1983).

Actinomycetes are a complex group of microorganisms, sometimes difficult to characterise and Goodfellow and Cross (1974) wrote "...the microbial ecologist does well to characterise his isolates even to generic level."
Actinomycetes are thought to be significant in breakdown of relatively complex polymers occurring in plant litter (Goodfellow and Williams, 1983). Rhodococci assimilate unusual compounds such as alicyclic hydrocarbons, nitroaromatic compounds, polycyclic hydrocarbons, pyridine and steroids (Tarnok, 1976 and Cain, 1981 in Sneath et. al., 1986) and have also been implicated in the degradation of lignin-related compounds (Eggeling and Sahm, 1980, 1981 and Rast et. al., 1980, in Sneath et. al., 1986). Streptomyces sp are also known to actively degrade lignin and cellulose (MacKenzie et. al., 1984; Crawford, 1978). Isolation of these genera from decaying alder leaves thus strongly suggests that bacteria play a not insignificant role in leaf litter degradation.

The ranges of microorganims isolated in the present study and that of Suberkropp and Klug can be compared by grouping genera into arbitrary categories based on divisions used in Bergey's Manual of Systematic Bacteriology vols. 1 and 2 (Krieg, et. al., 1984 and Sneath et. al., 1986) as shown in table 32. This shows that eight different groups of microorganisms were isolated in this study and 6 in Suberkropp and Klug's study. A greater range of organisms might have been expected from Suberkropp and Klug's study since they obtained isolates from both water and leaves. Use of only one isolation technique probably restricted the number of isolates obtained. Only organisms present on degrading alder leaves were isolated in the present study and the occurrence of a greater diversity is a direct reflection of the variety of techniques used.

Suberkropp and Klug divided their isolates into major and minor genera according to how frequently they were isolated from agar plates. Growth of organisms on an agar plate however does not provide sufficient evidence for conclusions to be drawn as regards the number of bacteria in a natural habitat, but merely the ability of some isolates to utilize the medium. Suberkropp and Klug also found that aquatic Hyphomycetes produced a more invasive array of cell wall degrading enzymes and they concluded that they were therefore more active in leaf degradation than bacteria. It is however doubtful that all of their bacterial isolates were leaf colonisers because they included river water in their isolations and water isolates would not have been

Study	DIVISION							
	Gram negative aerobic	Gram negative aerobic-not related to 1	Gram positive spore-former	Gram negative facul-tative anaerobic	Gliding bacteria	Coryne-form	Entero-bacteriaceae	Strepto-mycete
Harris's	Pseudo-monas	Alcali-genes	Bacillus	Aeromonas	Cytophaga	Rhodo-coccus	Escherichia	Strepto-myces
study	Xantho-monas						Klebsiella	
Suber-kropp &	Pseudo-monas	Acineto-bacter	Bacillus		Cytophaga	Arthro-bacter	Serratia	
Klug's study		Flavo-bacterium			Sporo-cytophaga			
		Alcali-genes			Flexi-bacter			
		Achromo-bacterium						

Table 32: Division of isolates obtained from degrading alder leaves (Harris, present study) and from river water, oak and pignut hickory (Suberkropp and Klug, 1976) into classes according to divisions in Bergey's Manual of Systematic Bacteriology vols. 1 and 2 (Krieg et. al., 1984; Sneath et. al., 1986).

expected to elaborate cell wall degrading enzymes. Furthermore, the one week incubation period would not have allowed for growth of slower growing organisms such as Actinomycetes which are known to actively degrade plant cell walls. Further investigation of the enzyme production of these species isolates should have taken this into account.

Estimates of the relative percentages of structural polysaccharide in dried alder leaf cell walls showed that they contained approximately 29,5% pectin, 5,4% lignin, 26,4% hemicellulose and 38,7% cellulose. These percentages could be expected to be slightly lower had they have been corrected for nitrogen and ash, and they would still correlate well with the ranges of structural polysaccharide published by Selvendran (1983). The hemicellulose and pectic polysaccharides, added together, make up a greater percentage of the structural polysaccharides in dried alder leaf cell walls. This is significant because published work has shown that a larger range of microorganisms elaborate enzymes which can attack hemicellulose and pectin (Fogarty and Kelly, 1983) and this could be one reason why alder leaves are processed in the relatively short time period of 6-8 weeks.

Results obtained from a survey of pectin-degrading enzymes by selected isolates showed that a range of enzymes active at acid, neutral and alkaline pH's were produced by these organisms. Sodium polypectate gel liquefaction and cup plate assays in sodium polypectate gel showed that Xanthomonas, Ps. cepacia/chloraphis and Cytophaga produced

enzymes active at alkaline pH only and that Erwinia produced enzymes active at neutral and alkaline pH. The assays also showed that pectin degrading enzymes had been produced by some of the other isolates tested, but the findings of one assay did not support that of the other. Results from both assays showed however that the pectinase activity was more evident at neutral and alkaline pH's which is indicative of pectinmethyl esterase and pectate lyase activity respectively.

Induction of pectate lyase activity in liquid medium and assay of supernatants showed activity in Xanthomonas, Cytophaga, Ps. fluorescens, Alcaligenes and Ps. putida in descending order of strength. Thus Xanthomonas and Cytophaga were shown to produce pectate lyases by the three assays used and this was considered conclusive evidence of production of this enzyme. The pectate lyase activity of Ps. fluorescens was comparable to that of a reference Cytophaga strain which was a known pectate lyase producer. Pectate lyase activities in Ps. putida and Alcaligenes supernatants were lower than any of the reference strains and the results from this assay were not supported by either cup plate or liquefaction assays so pectate lyase production by these two isolates was considered negligible.

Pectinmethyl esterase activity was not demonstrated by reduction of pH of pectin incubated with culture supernatants from pectinmethyl esterase inductions. This technique had previously been successfully used to demonstrate fungal pectinmethyl esterase production

(Chamier, 1980). It was thus expected that at least some of the organisms which had been shown to degrade sodium polypectate at neutral pH, or a pectinase producing reference strain might be shown to affect a drop in pH or pectin. These negative results would tend to suggest that either sodium polypectate, used in solid media assays, was unsuitable for assay of enzymes whose preferred substrate is pectin and that the results received at neutral pHs were misleading or that the assay of pH reduction was not sufficiently sensitive to detect the bacterial pectinmethyl esterases. This assay is complicated by the fact that the natural pH of pectin is 3 (C. May, H.P. Bulmer, Hereford personal communication) and that at higher pH's spontaneous hydrolysis causes reduction of pH and it would therefore seem unlikely that anything but the strongest enzymes would be detected by this assay.

Xylanases were produced by Aeromonas, both Bacillus sp tested and both Streptomyces sp.

Cellulose degrading enzymes were produced by both Streptomyces isolates. The cellulase produced by these organisms was extracellular as evidenced by positive reducing sugar assays using clarified culture supernatants.

Cellulase production by Cytophaga was expected but results were negative. This organism has frequently been reported to produce cellulase and in this study it was isolated from damp filter paper. It would appear that the conditions used for cellulase induction failed to induce enzyme production

by Cytophaga and that under the conditions provided, only Streptomyces spp produced cellulase. Complete cellulase breakdown by a single bacterium has rarely been reported, but it is known that bacteria are able to affect breakdown of cellulose in a synergistic fashion. The reasons for this are not well understood. The choice of substrate has also been shown to influence results (Rodriguez et. al., 1983; Smith, 1983).

This survey has shown that within a diverse group of organisms selected from 226 alder leaf isolates representatives of all major classes of structural cell wall degrading enzymes are produced. The activities of these enzymes have not been rigourously quantified since it is unlikely that their behaviour in pure culture fluid supernatant from in vitro induction mirrors that in vivo. However, within the limits of the techniques used the presence of the enzymes has been demonstrated which tends to suggest that the isolates selected for this work were a reasonably representative sample of the population. The effect of pure cultures of these isolates and a mixed culture of bacteria and the aquatic Hyphomycete Tricladium splendens on the dry weight of alder leaves was assessed in order that the contribution made by these organisms on an uncolonised leaf might be better understood. The culture flasks were incubated at 25°C because previous work had shown that the majority of bacterial isolates grew more actively at this temperature. These results showed that with the exception of Aeromonas, all isolates in pure

culture, T. splendens in pure culture and a culture containing a mixture of all bacterial isolates and T. splendens affected significant reductions in dry weights of alder leaves within 24 days when compared with an uninoculated control. Ranking of results obtained using Turkey's HSD shows that the greatest changes were affected by Streptomyces (grey), the mixture of bacteria and T. splendens, Cytophaga, Proteus, Ps. cepacia/chloraphis, Rhodococcus, Bacillus (spreading) and Ps. maltophilia.

An efficient reduction of dry weight of alder leaves was expected by the mixture of microorganisms because of the improved efficiency of combining different activities and also the positive effects of co-existence. Results show that the mixture of microorganisms affected the second largest reduction in dry weight. It is not unexpected that the dry weight reduction affected by one of the microorganisms tested was greater than the mixture since the enzyme efficiencies within a group would not be strictly additive. Some microorganisms would compete for the same polymer and a finite quantity of that polymer would restrict the amount of weight loss which would be recorded as a result of its breakdown. Furthermore, the artificiality of a pure culture system would not create competition and therefore all of the organisms' energy could be chanelled into metabolising the substrate. A mixed culture would however be expected to affect a highly efficient and also complete breakdown of the substrate whereas few organisms in

pure culture could affect total breakdown of a substrate as complex as a leaf.

A feature evident in ranking of results is that both Streptomyces (grey) and Cytophaga affected large reductions in dry weight of alder leaves. Streptomyces (grey) has been shown to produce an active cellulase and although cellulase production was not induced in pure culture of Cytophaga, it was isolated from filter paper and would therefore be expected to produce cellulase under the appropriate conditions. Streptomyces (white), also a cellulose producer affected a less significant reduction in dry weight of the leaves, possibly because its cellulase was weaker than Streptomyces (grey). These results nevertheless suggest that breakdown of cellulose in alder leaves accounted for a greater weight reduction than did xylanase or pectinase activity. This is possibly because without the activity of cellulases certain of the areas remain protected from attack by pectinases and xylanases, or that maceration of leaf tissue by pectinases and xylanase activity are slower processes or simply that a greater proportion of the leaf dry weight is made up of cellulose. Calculation of the percentage content of structural polysaccharide by weight does indeed show that cellulose makes up the greatest proportion of the cell wall structural polysaccharide thus supporting the third hypothesis.

Significant reduction in dry weight of leaves acted on by pure cultures of all isolates, many of which did not produce cell wall degrading enzymes in pure substrate assays,

suggests that they were able to produce these enzymes *in vivo* but that the artificial induction techniques failed to induce enzyme production. Dry weight reductions of alder leaves incubated with isolates not shown to produce cell wall degrading enzymes were similar to reductions affected by *Xanthomonas* and *Ps. fluorescens* both of which had produced pectinases in pure culture. Furthermore, the dry weight reduction caused by *T. splendens*, which was shown by Chamier (1980) to produce a powerful pectinase, is comparable to that of *Xanthomonas* and *Ps. fluorescens*. This suggests firstly, that structural cell wall degrading enzymes were produced by most of the isolates although artificial media failed to induce their production and secondly, by ranking of the reduction values, that the enzymes might have been pectinases.

Three features of dry weight reduction of alder leaves by isolates are also noteworthy. *Proteus* affected a highly significant reduction not expected by a member of the *Enterobacteriaceae*. Cup plate assays had however shown *Proteus* to produce pectinases at acid, neutral and alkaline pH. These results show that species isolated from the environment, although successfully characterised, should not always be expected to behave in textbook fashion. All pseudomonads affected significant reduction in dry weight, but the most significant reduction in dry weight was not affected by *Ps. fluorescens* which was the species most commonly isolated. This evidence serves as another example of the inaccuracy of assuming importance of an isolate

merely on the basis of frequency of isolation and culture. The aquatic Hyphomycete *T. splendens* affected a reduction in dry weight comparable to that affected by the pseudomonads tested, but not to the *Streptomyces* (grey) and *Cytophaga* which affected the greatest reduction in dry weights. This suggests that the efficiency of bacterial breakdown at least *in vitro*, is comparable to that of *T. splendens*.

Consideration of results obtained from the enzyme survey and from measurements of reduction in dry weights of alder leaves incubated with pure and mixed cultures of bacteria and *T. splendens* highlight the limitations of enzyme surveys per sé. These results have however been valuable as guidelines to assist in explaining observations made when the same organisms were placed under conditions which mimicked the natural environment more closely.

CONCLUSIONS AND FUTURE WORK

This work has addressed the presence of bacteria on degrading leaf litter in freshwater. It has shown that bacteria colonise degrading alder leaves in a distinct and recognisable pattern irrespective of river temperature in the River Bourne that a similar simplified colonisation pattern can be demonstrated *in vitro* with a known representative selection of the flora, that representatives of all classes of structural polymer degrading enzymes are produced by these bacteria and that most of the organisms isolated from decaying alder leaves affect a significant reduction in dry weight.

Morphology, enzyme production and *in vitro* colonisation by selected isolates has shown that the bacteria isolated from decaying alder leaves were representative of the flora *in vivo*. Direct observation has however shown the presence of certain morphotypes which were not isolated in this study. Clearly these organisms will not be isolated by conventional means and novel techniques and some imagination are called for in order to isolate these organisms. Isolates should also be characterised so that future work can be critically compared.

Bacterial colonisation of alder leaves *in vitro* observed under SEM by known and characterised isolates showed a colonisation pattern similar to that observed *in vivo*. The technique for culture, differentiation and enumeration of such colonised organisms was also described and

demonstrated, but preliminary investigation showed that in order to obtain an accurate record of colonisation this work would have to be replicated many times. Although this was beyond the scope of this project certain trends were observed eg increase in number of *Streptomyces* sp coincidental with loss of leaf integrity, which suggests that further investigation into this aspect of colonisation would give rewarding and meaningful results.

A survey of the enzymes produced by the bacteria showed that they produced the enzymes necessary for cell wall breakdown and that the majority affect a reduction in dry weight of leaves. Results also show that a mixture of microorganisms affect a greater reduction in dry weight of alder leaves than most isolates in pure culture. Little is known of the modes of action of enzymes elaborated by environmental bacteria such as those isolated in this study which are of limited pathological or clinical importance, although such knowledge would greatly assist in understanding the ecology of the individuals and collectively, of the flora. The logical progression of such an exercise is to study enzymatic breakdown of cell wall structural polymers by groups of representative microorganisms.

This work is intended to be complementry to that of Chamier (1980) whose thesis entitled "Pectinases in leaf degradation by aquatic Hyphomycetes" deals with the ecology of those fungi on alder and oak leaves in the River Bourne. Both this study and Chamier's have therefore investigated the roles of microorganisms on degrading leaf litter in one

river the present study from a bacteriological viewpoint and the previous one, a mycological viewpoint. Both studies have shown ordered colonisation patterns of those leaves and that the microorganisms produce the enzymes necessary for leaf breakdown. This evidence could be profitably combined and used to obtain a better understanding of the ecosystem and the interrelationships between the microflora which is, afterall, one of the fundamentals of microbial ecology.

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ADDENDUM

Deschamps, A.M., Mahoudeau, G., Lebeault, J.M. (1980)

Fast degradation of kraft lignin by bacteria.
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APPENDIX

ROW	Day1	Day2	Day3	Day4	Week1	Week2
1	13500000	14600000	12800000	9520000	13500000	14400000
2	14600000	25200000	18400000	18700000	28700000	11100000
3	17300000	33800000	25700000	28600000	38100000	14300000
4	21600000	39200000	39500000	35900000	50100000	15100000
5	25600000	43500000	45900000	43500000	60900000	13900000
6	11900000	9370000	9210000	9210000	19200000	11100000
7	17300000	16800000	14800000	15400000	31900000	14800000
8	23800000	29100000	22200000	24800000	42500000	12200000
9	34600000	36500000	33800000	34800000	50000000	13500000
10	43200000	40600000	41100000	41900000	64100000	93700000
11	31800000	46800000	10800000	12100000	10600000	19500000
12	8090000	4440000	20200000	19800000	19100000	12100000
13	12900000	12200000	29100000	28900000	30000000	8730000
14	18000000	23000000	38100000	36500000	42700000	16500000
15	22700000	34800000	46200000	41800000	49800000	7930000
16	5080000	40600000	17800000	12700000	8090000	19500000
17	7300000	7620000	26700000	25600000	13500000	19200000
18	10800000	13200000	36400000	34400000	18300000	12100000
19	16700000	19700000	43200000	46900000	30200000	11900000
20	19440000	26500000	51100000	56500000	42700000	13700000
21	3650000	32700000	9840000	8570000	14800000	15400000
22	6830000	6350000	18100000	14900000	24900000	10500000
23	10300000	12900000	25700000	24300000	31700000	12700000
24	13000000	17500000	33000000	33500000	42400000	15900000
25	17800000	27900000	40300000	40100000	60600000	5710000

ROW	Week3	Week4	Week5
1	3390000	3710000	2480000
2	4380000	3770000	3260000
3	2700000	1860000	2690000
4	2840000	2740000	2210000
5	3310000	3700000	2170000
6	3610000	4620000	3990000
7	3870000	4130000	4580000
8	4470000	4410000	4020000
9	5440000	3860000	2680000
10	3320000	5380000	5260000
11	2010000	3800000	*
12	3170000	3280000	*
13	2770000	3880000	*
14	4200000	2980000	*
15	3200000	4600000	*
16	2440000	2840000	*
17	3070000	5040000	*
18	2470000	3090000	*
19	2900000	4990000	*
20	3520000	5640000	*
21	3200000	5290000	*
22	3610000	3710000	*
23	3390000	3420000	*
24	5020000	4110000	*
25	4680000	4690000	*

Table 1: Individual counts of bacteria/cm^2 colonising alder leaves incubated in the River Bourne during run number 2.
*=missing data

ROW	day1	day2	day3	Week1	Week2	Week3	Week4
1	1350000	2950000	2470000	2990000	2580000	3630000	4690000
2	1650000	2790000	2710000	3320000	2160000	2390000	5810000
3	2010000	1980000	1990000	3020000	3430000	3780000	5780000
4	1120000	2840000	2260000	2430000	2630000	3110000	4290000
5	1030000	2790000	2290000	2430000	2880000	4110000	5830000
6	2050000	2580000	3840000	2320000	3220000	4570000	11900000
7	1580000	2040000	3240000	2900000	2720000	3490000	9450000
8	1860000	2950000	4560000	3050000	2610000	4470000	8200000
9	1190000	2010000	4350000	2820000	1530000	4850000	9840000
10	1890000	2090000	3750000	3090000	1950000	4370000	10600000
11	2470000	1850000	2850000	2260000	3150000	3150000	5430000
12	2190000	1440000	2600000	2590000	3030000	3310000	6060000
13	2240000	2190000	2440000	2360000	3150000	3900000	5390000
14	2270000	2140000	2040000	2490000	2370000	3420000	8790000
15	2590000	1950000	3110000	3410000	2480000	3690000	7090000
16	1970000	3090000	2890000	2630000	2240000	4460000	6330000
17	1510000	3640000	2510000	2480000	2360000	3390000	5790000
18	2060000	3020000	2410000	2680000	2900000	4640000	4470000
19	1290000	3480000	2950000	3580000	2190000	3620000	6480000
20	1610000	2310000	3910000	3190000	2240000	4260000	5230000
21	2210000	5220000	2280000	2460000	2060000	3250000	10300000
22	2570000	3170000	2670000	2590000	2710000	3540000	5620000
23	1530000	2690000	2560000	2410000	2510000	3040000	4520000
24	1960000	2640000	2160000	2250000	2890000	3940000	6220000
25	1690000	4280000	1980000	2090000	1820000	3410000	5480000

ROW	Week5	Week6	Week7
1	8060000	8910000	8090000
2	11000000	12300000	7280000
3	9030000	9080000	8770000
4	9340000	6350000	5310000
5	10700000	7080000	7940000
6	10500000	14000000	13400000
7	8090000	14800000	10700000
8	10500000	13800000	11300000
9	11600000	12600000	13900000
10	12400000	9720000	8660000
11	7970000	10100000	11100000
12	7750000	8670000	7770000
13	6330000	11300000	11500000
14	9220000	9330000	9420000
15	7500000	10300000	9220000
16	7730000	9420000	10300000
17	8900000	8560000	6910000
18	6610000	8130000	8050000
19	8480000	9170000	8220000
20	9480000	7470000	7440000
21	13200000	11900000	8950000
22	14800000	11300000	9580000
23	11400000	12100000	14200000
24	7970000	8500000	12100000
25	9170000	8560000	9730000

Table 2: Individual counts of bacteria/cm^2 colonising alder leaves incubated in the River Bourne during run number 5.

ROW	Day1	Day2	Day3	Day4	Week1	Week2
1	32800000	7140000	12100000	20800000	27600000	911000(
2	46800000	15200000	23000000	34900000	53200000	118000(
3	71100000	23300000	28300000	50500000	79400000	656000(
4	95100000	26500000	43000000	61300000	102000000	878000(
5	122000000	31400000	65100000	75200000	117000000	102000(
6	21300000	3970000	10600000	13000000	22500000	132000(
7	55100000	4760000	20900000	23300000	39800000	128000(
8	75400000	4760000	28100000	33800000	54900000	128000(
9	101000000	8410000	39700000	48300000	74400000	253000(
10	117000000	10600000	50300000	60000000	94300000	111000(
11	16700000	9520000	19000000	23300000	26200000	117000(
12	42200000	13000000	37500000	40000000	42200000	119000(
13	72200000	17300000	53500000	59100000	60900000	168000(
14	91900000	26000000	71300000	73700000	78900000	73300(
15	19000000	30800000	88200000	85900000	97500000	151000(
16	17600000	7940000	11100000	18300000	17500000	118000(
17	12500000	14800000	21100000	26500000	33900000	159000(
18	20900000	25400000	31900000	44900000	59700000	170000(
19	31300000	38100000	36500000	53300000	72500000	136000(
20	*	50300000	45600000	70500000	84900000	104000(
21	*	55600000	58900000	17600000	*	91100(
22	*	11800000	5240000	30200000	*	74400(
23	*	20000000	21400000	43000000	*	88800(
24	*	31100000	34100000	54800000	*	74400(
25	*	33900000	44900000	62500000	*	113000(

ROW	Week3	Week4
1	1040000	4720000
2	1160000	4210000
3	1410000	3100000
4	1400000	3930000
5	866000	4100000
6	788000	3980000
7	822000	4640000
8	511000	2860000
9	811000	5200000
10	678000	4820000
11	956000	4990000
12	822000	7410000
13	478000	2800000
14	667000	4500000
15	744000	3140000
16	989000	6180000
17	756000	3940000
18	656000	3530000
19	800000	3190000
20	444000	5590000
21	988000	4920000
22	1000000	4660000
23	900000	5600000
24	633000	6540000
25	633000	4830000

Table 3: Individual counts of bacteria/cm^2 colonising sterile alde
leaf discs incubated *in vitro* with river water.
*=missing data

Isolate or Mixture	Number	Weight of leaves in g after incubation
control		0,963
		0,970
		1,039
Streptomyces (grey)	225	0,49
		0,42
		0,46
Streptomyces (white)	226	0,68
		0,68
		0,60
Janthinobacterium	15	0,77
		0,69
		0,68
Ps. cepacia/chloraphis	179	0,69
		0,52
		0,52
Ps. maltophilia	210	0,70
		0,54
		0,58
Ps. testosteroni	197	0,80
		0,66
		0,64
Ps. fluorescens	163	0,65
		0,80
		0,54
Ps. putida	166	0,69
		0,52
		0,66
Aeromonas	174	0,82
		0,67
		0,75
Bacillus (spreading)	201	0,56
		0,76
		0,52
Bacillus (discrete)	149	0,71
		0,61
		0,65
Alcaligenes	168	0,81
		0,75
		0,61

Isolate or Mixture	Number	Weight of leaves in g after incubation
Erwinia	169	0,73
		0,73
		0,58
Aeromonas	174	0,76
		0,65
		0,83
Rhodococcus	155	0,73
		0,53
		0,54
Proteus	219	0,57
		0,59
		0,48
Cytophaga	222	0,60
		0,45
		0,52
Xanthomonas	193	0,62
		0,62
		0,72
Tricladium splendens		0,59
		0,67
		0,63
Bacteria & T. splendens		0,48
		0,50
		0,47

Table 4: Weight in grams of alder leaves after 24 days incubation with microorganisms. Starting weight 1 g

A critical comparison of the use of standard morphological and biochemical testing, commercially available test strips and fatty Acid Profiling as a means of determination of large numbers of environmental bacteria

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INTRODUCTION

The action of microorganisms and macroinvertebrates together with the leaching of minerals plays an important role in decomposition of litter in small heterotrophic streams,[1,14]. Several authors have recognised and documented that fungi, particularly the aquatic Hyphomycetes, and bacteria are important intermediaries in this food chain,[6,13].

Characterisation and determination of bacteria involved in leaf decay is however limited largely because of the time consuming nature of the work and the degree of subjectivity in interpretation of the results. In order to gain a better understanding of the role played by bacteria in detritus food chains it is important that the microbial ecologist is familiar with the different groups of organisms with which he is working and that the work is repeatable and therefore comparable to other work of this nature.

Conventional methods for determination of bacteria place much emphasis on morphological and biochemical characteristics. These were the only methods available to early microbiologists and remain, for the most part, guidelines for present day characterisation. The methods do not facilitate determination of taxa which show variability for any given character and as more bacteria are isolated from different sources, variation increases. The microbial ecologist, who generally deals with large numbers of isolates including many perhaps undescribed taxa is thus confronted with a formidable task if he wishes to identify these organisms.

This work aims to compare critically the use of standard morphological and biochemical criteria in the characterisation of bacteria from the surfaces of alder (Alnus glutinosa) leaves in a freshwater stream with results obtained both from commercially available identification strips which make use of a standard set of biochemical tests, and gas liquid chromatograph analysis of cell fatty acids.

Fatty acid profiling has been shown to be a rapid accurate method for specific and subspecific determination of many plant pathogenic bacteria (unpublished resullts) and other bacteria[8,9].

MATERIALS AND METHODS

Isolation of bacteria

Organisms were removed from the surfaces of alder leaves which had been incubated in a small freshwater stream for two weeks by paddling in a stomacher (A. J Seward Lab-Blender model BA 6021) for 5 minutes in order to produce a suspension of mciroorganisms[3]. The suspension was diluted, as appropriate, in sterile Ringers solution and plated out onto broad spectrum bacteriological media or onto selective media viz: chitin agar[7], pectin agar[4], leaf homogenate agar (KC 1.7g; $Na_2HPO_4.12H_20$ 4,1g; $MgSO_4$ $7H_20$ 0.05g; $CaCO_3$ 0.02g; $FeSO_47H_20$, 0.1g; ground alder leaves 0,1g; agar 15g; distilled water 1000 ml; pH adjusted to 7,2 with $NaCO_3$ after autoclaving), peptone succinic acid agar (Hylemon et al, 1973) and oatmeal agar[12].
Incubation temperature and duration varied according to which genera were sought.

Reference cultures were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB).

Determinative tests

i) Morphological, nutritional and physiological tests.

Following isolation and purification a total of 30 isolates were processed through the following battery of tests[10]: morphology (viewed under phase contrast on a Leitz Diaplan microscope), Gram stain, catalase production, oxidase production, motility, glucose metabolism, production of fluorescent pigments and indole production.

Isolates were categorised on the basis of results obtained from the above tests. Further testing as appropriate, involved gelatin liquefaction, transmission electron microscopic viewing of flagella number and insertion, carbohydrate fermentation and assimilation[10] and pectate lyase production[15].

ii. API test strips

Gram negative, oxidase positive isolates were also inoculated onto commercially available test strips such as API 20NE strips (API Laboratory Products Ltd, Basingstoke, Hants, UK) according to manufacturers instructions.

iii. Fatty acid profiles

Cellular fatty acid profiles were obtained for all isolates.

Bacteria were cultured on sterile freshly prepared plates of trypticase soy agar (BBL Trypticase Soy Broth 30g, Difco Bacto agar 15g, distilled water 1 litre) at 28°C for 24 $\pm$ 1 hour. Cells were harvested from areas of confluent growth just prior to colony separation, usually from the third of four dilution streaks. Cells (approx. 5 mm^3) were removed with a sterile aluminium spatula into small screw-capped test tubes with teflon lined caps. Cell lipids were saponified by the addition of 1 ml of sodium hydroxide/

methanol solution (NaOH pellets 45 g, methanol 150 ml, deionised distilled water 150 ml). Samples were placed in a boiling water bath for 30 minutes. Tubes were vortexed before and once during boiling.

Fatty acids were methylated by the addition of 2ml hydrochloric acid/methanol solution (6N HCl 325 ml, methanol 275 ml). Samples were heated in a water bath at $80^{o}C \pm 1^{o}C$ for 10 minutes and immediately cooled to $<20^{o}C$.

Fatty acid methyl esters (FAMEs) were extracted in 1.25 ml of hexane/ ether solution (hexane 200 ml, tertbutyl methyl ether 200 ml). Tubers were rotated for 10 minutes. The aqueous layer was discarded and the FAMEs washed in 3 ml sodium hydroxide solution (NaOH pellets 10.8 g, deionised distilled water 900 ml) for 5 minutes. All reagents used were HPLC grade.

FAMEs were separated by Gas chromatography by injection of 1 ul into a 25 m fused silica capillary column coated with methyl phenyl silicone on a Hewlett Packard 5890A Gas Chromatograph using hydrogen as the carrier gas. Injection was standardised using a Hewlett Packard 7673A Automatic Injector. FAMEs were detected by flame ionisation and peaks integrated on a Hewlett Packard 3392A integrator. FAME identification, FAME profile generation and subsequent culture identification by comparison with a library of aerobic bacteria were made using Hewlett Packard 5898A Microbial Identification System software package 19298A.

A FAME calibration mixture contained 9 carbon - 20 carbon straight chain saturated esters and general -2 and -3 hydroxy esters as qualitative and quantitative standards respectively. This was obtained from Microbial I.D. Inc., Suite 115 Barksale Professional Centre, Barksdale Road, Newark, Delaware 19711, USA.

GC paramneters are given in Table 1.

TABLE 1

GC PARAMETERS FOR SEPARATION OF FAMES

Carrier Gas	Hydrogen	30 ml/min (FID)
		55 ml/min (split vent)
		5 ml/min (septum purge)
Auxilliary gas	Nitrogen	30 ml/min (FID)
		40 ml/min (trap purge)
	Air	400 ml/min (FID)
Split ratio	100:1	
Initial temperature	$170^{o}C$	
Initial time	0 min	
Programme rate	$5^{o}C/min$	
Final temperature	$270^{o}C$	
Final time	0 min	
Injection part temperature	$250^{o}C$	
FID temperature	300°	

RESULTS

Conventional nutritional and physiological tests allowed tentative assignment of 11 of the 30 isolates to genus (Table 2). Test strip analysis (API 20 NE) of all gram negative oxidase positive bacteria allowed generic determination for a further seven isolates.

Fatty acid profiling served three purposes: as a means of comparison with results obtained using the conventional morphological and biochemical methods, to identify those organisms displaying characteristics inappropriate for identification using API 20 NE test strips and to characterise and group those for which no determination was made. Fatty acid profiling gave a library match at specific level for 27 of the 30 isolates tested, whereas of the 13 gram negative oxidase positive isolates, 11 were identified by API 20 NE test strips (Table 3). Both these systems gave some measure of similarity to the library profile. The fatty acid profiling software gave a series of taxa with decreasing similarity indices. Table 3 includes only the first choice and the similarity index for that choice. Test strip matches were based on the percentage accuracy of the nutritional profile.

Of the 11 isolates (1-11) which were identified by both systems, 7 were in agreement of specific level. This included Xanthomonas maltophilia, recently reclassified from Pseudomonas and named differently by the two systems. Of the 5 for which there was no agreement even at generic level (isolates 9-13), initial results from conventional nutritional and physiological tests indicated that fatty acid profiling was probably more accurate for isolates 9 and 12 and that test strip analysis was probably more accurate for isolate 11 (Pseudomonas paucimobilis). This taxon is not included in the fatty acid profile library. Of the remaining 21 isolates which were identified by fatty acid profiling, 5 were identified as Cytophag johnsonae but with low

similarity indices, 12 as members of the *Enterobacteriaceae*, 2 as *Bacillus* spp. and 2 as *Rhodococcus rhodochrous*.

Identification of the reference strains (Table 4) was generally accurate with both systems used. Eight of the 9 strains were correctly identified by fatty acid profiling as opposed to 6 by test strip analysis.

DISCUSSION

When identifiying large numbers of microorganisms as in an ecological study the time spent identifying, the availability and cost of equipment and media, and accuracy are important considerations.

The biochemical and morphological characteristics selected for initial characterisation of alder leaf surface bacterial isolates are routinely used for this purpose and the equipment and media were therefore readily available. The factor limiting the use of a broader range of biochemical tests is time. The cost of processing large numbers of isolates is difficult to quantify since there is usually no large capital outlay required and routinely available media are used. Generally the cost of the media is far outweighed by the time taken to prepare the media, perform tests and then to dispose of, or wash contaminated equipment.

Selection of such consecutive tests is dependent upon resullts obtained and their interpretation. Accuracy of identification is thus very dependent upon experience. Few ecologists have the necessary relevant diagnostic experience for this type of identification.

The use of commercially available test strips to assist in identification of Gram negative, oxidase positive organisms helped reduce the number of isolates which required further biochemical testing in order to facilitate identification. Additionally the test strips required no specialised equipment and results were obtained within 24 to 48 hours. The cost of one Test Strip is £1.70 and a reagent kit £4.40 which can be used for 125 Strips.

Fatty acid profiling requires relatively little expertise in bacterial taxonomy or selection of determinative tests and the interpretation of results obtained from them. Accuracy of identification is generally excellent for most groups of bacteria. The process can be semi-automated so that 50 determinations can be made in approximately 50 hours. Stead (1988b) demonstrated that accuracy of determination at specific level of more than 400 reference strains of plant pathogenic bacteria from the National Collection of Plant Pathogenic Bacteria (NCPPB) was approximately 98%.

In our study of 8 reference strains from the NCPPB selected because they were in the API 20 NE library, 7 were correctly determinated at specific level by the API 20 NE test strips and by fatty acid profiling. Of the 9 reference strains included in the fatty acid profiling library, 8 were correctly identified by fatty acid profiling. Of the alder leaf isolates tested the fatty acid profiling software named 90% of the isolates. The low similarity indices for some of these indicates that the name given is perhaps not wholly accurate but is the closest fit from within the same group of bacteria. The higher the index the more close the fit of the test profile with that of the selected reference library determination. Values greater than 0.5 on a 0 - 1.0 scale are usually indicative of accurate determination at specific level. Values above 0.1 - 0.2 are usually indicative of accurate determination at generic level. This type of information is valuable and becomes more

accurate and useful as the number of taxa in the library increases. Reference to standard texts such as Bergey's Manual of Systematic Bacteriology will enable selection of a few key tests to determine taxa more accurately where similarity indices obtained are low or when several choices with similar indices are given. Identification by any profiliing method can only be as good as the quality of the libraries developed. In both cases libraries used are commercially available.

Qualitative and quantittive composition of bacterial fatty acids provide stable phenotypic characters and even when specific determination cannot be made the profile can be used to classify the organism to some extent[8,9,11]. This type of information is rarely available with test strips where best matches may include several unrelated taxa. Results with test strips may be further complicated by inconclusive results or problems with contamination. Fatty and profiling can be affected by changes in cultural conditions such as medium composition, culture age, temperature and oxygen status. For plant pathogenic Pseudomonas species incubation time and temperature did not significantly affect determination at specific level (unpublished results). However it is important that library development and testing should be made using defined standard cultural conditions. Software is also available for generation of libraries of specific groups of bacteria not included in the Hewlett Packard library.

This work has found cellular fatty acid profiling to be a rapid, accurate and versatile method for determination of large numbers of unknown bacteria. Standard morphological and biochemical testing and use of test strips although more readily obtained, are useful only for a restricted range of organisms.

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TABLE 2: Categorisation of isolates from the surface of alder leaves incubated in a freshwater stream for 2 weeks, on the basis of standard biochemical and morphological testing.

Isolate Number	
1	Pseudomonadaceae
2	Pseudomonadaceae
3	Vibrionaceae
4	Pseudomonadaceae
5	Xanthomonas sp.
6	Gram negative aerobic rod
7	Xanthomonas sp.
8	Gram negative aerobic rod
9	Pseudomonadaceae
10	Escherichieae
11	Pseudomonadaceae
12	Janthinobacterium
13	Gram negative rod
14	Coryneform
15	Klebsiella sp.
16	Cytophaga sp.
17	Pseudomonadaceae
18	Bacillus sp.
19	Cytophaga sp.
20	Cytophaga sp.
21	Cytophaga sp.
22	Cytophaga sp.
23	Bacillus sp.
24	Coryneform
25	Xanthomonas
26	Gram positive aerobic rod
27	Enterobacteriaceae
28	Enterobacteriaceae
29	Enterobacteriaceae
30	Enterobacteriaceae

Table 3: Identification of isolates from the surface of alder leaves incubated in a freshwater stream for 2 weeks, by APl 20 NE test strips and fatty acid profiling.

Isolate Number	FA Profile Identity	Similarity Index	Test Strip Identity	Accuracy %
1	Pseudomonas testosteroni	.78	Pseudomonas testosteroni/ alcalisgenes	64
2	Pseudomonas fluorescens	.84	Pseudomonas fluorescens	99
3	Aeromonas hydrophila	.36	Aeromonas hydrophila	99
4	Pseudomonas putida	.52	Pseudomonas putida	99
5	Xanthomonas maltophilia	.61	Pseudomonas maltophilia	99
6	Pseudomonas testo	.83	Pseudomonas testosteroni/ alcaligenes	99
7	Xanthomonas maltophilia	.59	Pseudomonas maltophilia	99
8	Alcaligenes faecalis	.31	Alcaligenes denitrificans	70
9	Pseudomonas putida	.40	Alcaligenes denitrificans	D
10	Salmonella typhimurium/ typhi	.67	Aeromonas hydrophila	D
11	Pseudomonas paucimobilis	.68	Pseudomonas paucimobilis	90
12	Janthinobacterium lividum	.95	Unacceptable profile	
13	Proteus vulgaris	58	Unacceptable profile	
14	Rhodoccus rhodochrous	24	N/A	
15	Klebsiella terrigena	.73	N/A	
16	Cytophaga johnsonae	.17	N/A	
17	No match		N/A	
18	Bacillus thuringiensis	.56	N/A	
19	Cytophagae johnsonae	.06	N/A	
20	Cytophaga johnsonae	.09	N/A	
21	Cytophaga johnsonae	.04	N/A	
22	Cytophaga johnsonae	.08	N/A	
23	Bacillus mycoides	18	N/A	
24	Rhodoccus rhodochrous	55	N/A	
25	No match		N/A	
26	No match		N/A	
27	Serratia plymuthica	65	N/A	
28	Enterobacter intermedium	63	N/A	
	Klebsiella terrigena	62		
	Erwinia chrysanthemi	60		
29	Escherichia coli	64	N/A	
	Enterobacter agglomerans	61		
	Erwinia herbicola	49		
30	Escherichia coli	64	N/A	
	Salmonella typhimurium/typhi	64		
	Enterobacter agglomerans	54		

TABLE 4

Identification of Standard reference strains of bacteria by Test Strips and fatty acid profiling

Reference strain identity	Test Strip Identity	Accuracy %	Fatty Acid Profile Identity	Similarity Index
Pseudomonas fluorescens NCPPB 1964	Pseudomonas cepacia	96	Pseudomonas fluorcescens	0.571
Pseudomonas aeruginosa NCPPB 1965	Pseudomonas aeruginosa	96.3	Pseudomonas aeruginosa	0.765
Pseudomonas syringae pathovar syringae NCPPB 281	Aeromonas salmonicida	99.9	Pseudomonas syringae	0.990
Xanthomonas maltophilia NCPBB 1974	Pseudomonas maltophilia	99.4	Xanthomonas maltophilia	0.882
Pseudomonas acidovorans NCPBB 1967	Pseudomonas acidovorans	99.4	Pseudomonas acidovorans	0.621
Pseudomonas putida NCPBB 1806	Pseudomonas putida	99.8	Pseudomonas tolassi	0.369
Pseudomonas cepacia NCPBB 1962	Pseudomonas cepacia	99.9	Pseudomonas cepacia	0.611
Pseudomonas alcaligenes	Pseudomonas testosteroni /alcaligenes	90.7	Pseudomonas alcaligenes	0.440
Xanthomonas campestris pathovar campestris NCPPB 528	N/A		Xanthomonas campestris	0.570