APPLICATION OF IMAGE ANALYSIS IN MICROECOPHYSIOLOGY RESEARCH:

METHODOLOGY DEVELOPMENT

B. T. DUDLEY BSc (Hons)

Submitted in fulfilment of the academic requirements for the degree of Master of Science in the Department of Microbiology and Plant Pathology, University of Natal, Pietermaritzburg.

1998

ABSTRACT

Rehabilitation of landfill sites is important for successful land utilization. Revegetation is one key element of the process since it can overcome aesthetic problems. The inimical challenges of landfill leachate and gas are largely responsible for the difficulties associated with the revegetation of completed sites. Many components of landfill leachate can be catabolized by microbial associations thereby reducing their impacts on the environment. The importance of research on interactions between pollutants, microorganisms and soil is its applicability in environmental risk assessment and impact studies of organic pollutants which enter the soil either accidentally or intentionally.

The application of image analysis with microscopy techniques to landfill soil-pollution interactions provides a means to study surface microbiology directly and to investigate microbial cells under highly controlled conditions. This research focused on the development of a method to study the real time processes of attachment, establishment, growth and division of microbial cells/associations in site covering soils. Image analysis provides a powerful tool for differential quantification of microbial number, identification of morphotypes and their respective responses to microenvironment changes. This minimal disturbance technique of examining visually complex images utilizes the spatial distributions and metabolic sensitivities of microbial species. It was, therefore, used to examine hexanoic acid catabolizing species, both free-living and in a biofilm, with respect to obviating the threat of hexanoic acid to reclamation strategies.

The three sources of inoculum (soil cover, soil from the landfill base liner and municipal refuse) were compared for their ability to provide associations which catabolized the substrate rapidly. During the enrichment programme the inocula were challenged with

different concentrations of hexanoic acid, a common landfill intermediate. From the rates at which the substrate was catabolized conclusions were drawn on which concentration of hexanoate facilitated the fastest enrichment. The results of initial batch culture enrichments confirmed that the soil used contained microbial associations capable of catabolizing hexanoic acid at concentrations < 50mM, a key leachate component. Exposing the landfill top soil microorganisms to a progressive increase in hexanoic acid concentration ensured that catabolic populations developed which, *in situ*, should reduce the phytotoxic threat to plants subsequently grown on the landfill cover.

The analysis of surface colonization was simplified by examining the initial growth on newly-exposed surfaces. The microbial associations generated complex images which were visually difficult to quantify. Nevertheless, the dimensional and morphological exclusions which were incorporated in the image analysis software permitted the quantification of selected components of the associations although morphology alone was inadequate to confirm identification.

The effects of increasing the dilution rate and substrate concentration on the growth of surface-attached associations in Continuous Culture Microscopy Units (CCMUs) were examined. Of the five dilution rates examined the most extensive biofilm development (9.88 μm^2) during the selected time period (72h) resulted at a dilution rate of $0.5h^{-1}$ (at 10mM hexanoic acid). The highest growth (608 microorganisms.field^{-1}) was recorded in the presence of 50mM hexanoic acid (D = $0.5h^{-1}$). To ensure that the different morphotypes of the associations were able to multiply under the defined conditions a detailed investigation of the component morphotypes was made. Numerically, after 60h of open culture cultivation in the presence of 50mM hexanoic acid, rods were the predominant bacterial morphotypes (43.74 field^{-1}) in the biofilms. Both rods and cocci

were distributed throughout the CCMUs whereas the less numerous fungal hyphae (0.25 field^{-1}) were concentrated near the effluent port.

The specific growth rates of the surface-attached associations and the component morphotypes were determined by area (μm^2) colonized and number of microorganisms.field^{-1} and compared to aerobic planktonic landfill associations. From area determinations ($>$ 0.16 h^{-1}) and the number of microorganisms.field^{-1} 10mM hexanoic acid was found to support the highest specific growth rate ($>$ 0.05 h^{-1}) of the surface-attached association isolated from municipal refuse. With optical density determinations, the highest specific growth rate (0.01 h^{-1}) was recorded with 25mM hexanoic acid. The surface-attached microbial associations component species determinations by area and number showed that the hyphae had the highest specific growth rate ($>$ 0.11 h^{-1}). The surface-attached microbial association specific growth rate determinations from the discriminated phase (0.023 h^{-1}), area colonized (0.023 h^{-1}) and number of microorganisms (0.027 h^{-1}) calculated from the results of the component species rather than the association should give more accurate results.

The specific growth rate obtained differed depending on the method of determination. Any one of these may be the "correct" answer under the cultivation conditions. Depending on the state (thickness) of the association (free-living, monolayer or thick biofilm) the different monitoring methods may be employed to determine the growth. As a consequence of the results of this study, the kinetics of microbial colonization of surfaces *in situ* may be subjected to the same degree of mathematical analysis as the kinetics of homogeneous cultures. This type of analysis is needed if quantitative studies of microbial growth are to be extended to surfaces in various natural and artificial environments.

DECLARATION

I hereby certify that this research, unless specifically indicated in the text, is the result of my own investigations.

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B.T. DUDLEY

1998

ACKNOWLEDGEMENTS

This research was funded by the Foundation for Research Development (FRD), the Department of Environment Affairs and Tourism , the Institute of Waste Management (Southern Africa), the University of Natal and Waste-tech (Pty) Ltd whose support is gratefully acknowledged.

This thesis would not have been possible without the help and support of numerous people, to whom my appreciation is extended:

My supervisors Prof. Mike Wallis, Prof. Eric Senior and Tony Bruton for their advice, support and encouragement.

The Centre for Electon Microscopy staff, Vijay, Belinda and Priscilla, for their cheerfully given advice and assistance.

The laboratory technicians of the Department of Microbiology and Plant Pathology, Cheryl Crooks, Ingrid Schlosser and Diane Fowlds.

Paul Forder (Glass Blowing Workshop) and Daryl Leibbrandt and the "guys" at the Mechanical Instrument Workshop, University of Natal, Pietermarizburg.

Prof. J. da Graca.

Petro Nortje.

Ray Lombard (Lombard and Associates).

Fellow postgrad's and friends in the Department of Microbiology and Plant Pathology for their advice and help.

Finally, and most importantly, my family, colleagues, friends and Natasha Williams for their much needed emotional support and encouragement throughout the course of this work.

CONTENTS

CHAPTER ONE

1. INTRODUCTION

1.1 *THE FUNCTION OF COVERING SOILS IN LANDFILL SITE REHABILITATION*

The inimical challenges of landfill leachate and gas are largely responsible for the difficulties associated with the re-vegetation of completed landfills (Arif and Verstraete, 1995). Numerous components of landfill leachate can be catabolized by microbial associations to reduce their impacts on the environment (Campbell, Parker, Rees and Ross, 1983). Biological and biochemical investigations of soil (Paul and Voroney, 1980) have provided information about the potential of leachate contaminated soils to support the productive growth of plants (Grandt, 1978; Power, Ries, and Sandoval, 1978).

Landfill, which is the most common method of refuse disposal uses soil as a container (Weeks, Mansell and McCallister, 1992), covering material (Robinson, Handel and Schmalhofer, 1992), renovating agent (Hasselgren and Christensen, 1992) and growth medium for the rehabilitation of the site (Chan, Davey and Geering, 1978). In this respect it is important to recognize that soil provides surface area which is often the medium for interactions between microorganisms and pollutants (Fontaine, Lehmann and Miller, 1991). The surface area per unit volume of soil greatly exceeds that of the refuse in the landfill (Fuller, 1980; Scott, Wolf and Lavy, 1982). It is, therefore, reasonable to assume that the soil will have a significant influence on both the microorganisms and the catabolic products migrating from the refuse mass (Albrechtsen, 1994). Unfortunately, studies of the effect of surface attachment on growth and activity of bacteria in soil are difficult (Jenkinson and Ladd, 1981) and soil-microorganism-pollutant interactions have mainly been examined in soil columns (Webb, Phelps, Bienkowski, Digrazia, Reed, Applegate, White and Sayler, 1991). One of the shortcomings of column and *in situ* studies is that the identity and population size of the specific microbial component affecting or influencing attenuation are usually unknown and cannot readily be determined (du Plessis, Senior and Hughes, 1994).

The design of a final cover system is a very important aspect of the closure of sanitary landfills. Soil material properties and vegetation are important features (Weeks *et al.*, 1992). The key to understanding how landfill soil cover influences leachate attenuation

is undoubtedly to elucidate the extremely complex interactions between the different metabolic groups of organisms present. To determine the significance that covering soils (daily, intermediate and final) play in landfill leachate attenuation and subsequent revegetation a thorough understanding of the fundamental microbiology and biochemistry of the operative catabolic mechanisms is required (McGinnies and Nicholas, 1980). As few definitive microbiological and biochemical studies have been made on the complex interspecies interactions involved, a complete understanding remains unachieved. Two microbial population types must be recognized: the growth rate-dependent (free-living) species and the growth rate-independent (surface-attached) species, and the relative contributions of each must be resolved. A possible solution to the problem lies in the use of realistic models maintained under defined and controlled conditions (Parkes and Senior, 1988). Since many environmental biotechnologies are, characteristically, heterogeneous, such processes cannot be effectively examined under simple homogenous conditions such as monoculture bacterial study (Senior, 1991).

1.2 *MICROBIAL ASSOCIATIONS OF LANDFILLS*

One of the significant features of environmental biotechnology is the presence of microbial associations which arise from the utilization of multiple electron donors and, sometimes, multiple electron acceptors (Rittmann and Manem, 1992). A biofilm consists of cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin. It is a surface accumulation which is not necessarily uniform in time or space. Multiple-species biofilms comprise mixed microbial populations within an extracellular polymeric matrix bound to a substratum (Sutherland, 1977; Costerton, Irvin and Cheng, 1981; Bryers, 1993). These adherent associations are subject to interactions such as symbiosis or competition for space or a common substrate (Wolfaardt, Lawrence, Robarts, Caldwell and Caldwell, 1994). Such interactions are directly or indirectly influenced by a myriad of variables associated with the surrounding environment (Gilbert, Evans and Brown, 1993). Spatial distributions of microbial populations are constantly changing under the selection pressures exerted by processes such as: 1. Exchange of bacterial species with the bulk liquid phase; 2. Efficiency of metabolic conversion of limiting substrate(s) to viable cell mass and non-viable extracellular polymers; 3. Transport of limiting substrate and essential nutrients by molecular, convective transfer mechanisms and cell-free channel-like structures of variable sizes which interconnect the surface film

with the deep inner layers and appear to increase the biological surface area per unit volume ratio, which may facilitate transport of substrates into and waste products out of deep regions of the biofilm at rates greater than possible by diffusion alone (Massol-Deyà, Whallon, Hickey and Tiedje, 1995); and 4. Biofilm removal processes by physico-chemical mechanisms (sloughing) or as a result of prevailing hydrodynamics (shear-related detachment) (White, 1986; Downes, Lake and Schreiber, 1995). Wolfaardt *et al.* (1994) found that unique spatial relationships which developed among members of the community were distinctive in biofilms grown on chlorinated ring compounds such as diclofop. The same relationships did not develop when the biofilms were grown on more labile substrates but were conserved when the biofilms were cultivated with other chlorinated ring compounds. These unique consortial relationships indicated that syntrophic interactions may be necessary for optimal degradation of the numerous compounds in a landfill environment. Evolution of the microbial population spatially within a biofilm with reference to a particular function (e.g. self purification of contaminated surface- or ground-waters (Dudley, Lees, Bruton, Senior and Wallis, 1992a), *in situ* bioremediation of xenobiotics (Wolfaardt *et al.*, 1994), specific wastewater treatment systems (Beeman and Suflita, 1987) and landfill environments (Weeks *et al.*, 1992; Albrechtsen, 1994) will affect the overall functioning of the biofilm in the specific biological conversion (Hitchcock, Glasbey and Ritz, 1996). Consequently, it is critical to know how the ever-changing adherent population distributions affect overall system performance to better understand and operate biofilm systems.

Landfilled municipal refuse is rich in microorganisms (Filip and Küster, 1979; Westlake, Archer and Boone, 1995). Isolation of these microorganisms by standard cultural techniques has various disadvantages such as quantitative underestimation and selective inhibition or stimulation due to the cultivation conditions (Busscher and Van Der Mei, 1995). Furthermore, these techniques give little insight of the morphology of the indigenous microflora (Smed-Hildmann and Filip, 1988). Recognizing that most landfills are solid-state anaerobic digesters, a fundamental understanding of this principal aspect of refuse stabilization is required (Pohland and Al-Yoosfi, 1994). The degree of microbially-mediated stabilization of landfilled solid waste is a function of many site-specific variables, including waste characteristics, climate and construction and operation protocols (Pohland, Al-Yoosfi, Hanaki and Vasuki, 1994). Species composition is an important determinant of biofilm thickness and structure (Murga, Stewart and Daly,

1995). Unfortunately, detailed knowledge of the microbial associations involved is particularly lacking.

1.3 *SURFACE MICROENVIRONMENT INVESTIGATIONS*

To date, possibly due to methodological difficulties, few studies of the roles of soil surfaces have been reported despite the fact that knowledge of these is central to understanding the importance of microorganisms in revegetation (Jones and Mollison, 1948; Waid 1984; Evans and Ahlert, 1987). The complex nature (genotypical, structural and dynamic) of the municipal solid waste landfill environment in which bacteria proliferate has prompted microbial ecologists to develop experimental systems to study the behaviour of microorganisms under well-defined and controlled conditions (Wimpenny, 1992). Previous studies of population dynamics within surface microenvironments were limited by a lack of analytical methodology for estimating the growth of individual cells and microcolonies (Harvey and Young, 1980; White, 1983).

Albrechtsen (1994) determined the relationship between particle size fractions and numbers of viable bacteria and their activity for aquifer sediment sampled near a landfill site. Except for the finest fraction, good correlations were obtained between bacterial number and activity and particle surface area. It was concluded that quantitative sampling of the microbial populations of aquifers must include sampling of the sediment using techniques that retain pore water (Albrechtsen, 1994).

To determine the extent to which growth rate-independent microorganisms are involved, the communities must be examined in relatively undisturbed tracts. If a biofilm is disrupted essential information is lost (Moller, Kristensen, Poulsen, Carstensen and Molin, 1995). Unfortunately, the heterogeneity and complexity of the biofilm-forming associations makes quantification of colonization and changes in response to leachate perturbations difficult (Wilson and McNabb, 1983).

Considerable interest has been generated towards gaining a more complete understanding of the differences between the metabolic functioning of bacterial cells adsorbed onto a solid surface and those existing in a freely suspended state (ZoBell, 1943; Meinders, Mei, Busscher and Van Der Mei, 1995). This could help explain natural processes in the soil

(Daniels, 1980). Adhesion of microorganisms to surfaces has been found to influence metabolic activity and, therefore, catabolic capabilities (biodegradation) in many instances (Fletcher, 1985; Bar-Or, 1990; Van Loosdrecht, Lyklema, Norde and Zehnder, 1990). Adsorption of specific species may be an important factor in obtaining catabolic microbial associations where high concentrations of molecules in the aqueous phase are toxic/inhibitory to the potential degraders (Kefford, Kjelleberg and Marshall, 1982; Brown and Gilbert, 1993). Conversely, in some situations species attachment to surfaces may be a disadvantage. The result often depends on the nature of the organism, the type and concentration of the substrate, and the nature of the solid surface.

1.4 *IMAGE ANALYSIS IN MICROBIAL ECOLOGY*

A central problem in microbial ecology is the understanding of microbial growth and behaviour *in situ* (Costerton *et al.*, 1981; Lorenz, Aardema and Krumbein, 1981). Many structural studies of microbial biofilms have relied on light and electron microscopy (Eighmy, Maratea and Bishop, 1983). Problems associated with these techniques include disruption of biofilm structure during removal from the substratum (Robinson, Akin, Nordstedt, Thomas and Aldtich, 1984), laborious preparations (Lappin-Scott, Jass and Costerton, 1993) and extensive sample processing (Costerton, Cheng, Geesey, Ladd, Nickel, Dasgupta and Marrie, 1987) which introduce a range of artifacts. The phenomenal phenotypic plasticity of bacteria (Kuhn and Starr, 1970) places an especially high value on direct observations (Costerton *et al.*, 1987) or measurements (Caldwell, Kober and Lawrence, 1993) of the activity of bacterial cells growing in the system of interest (Van Houtte, Pons, Thomas, Louvel and Vivier, 1995). Light microscopy used in combination with computers is an effective tool but it is best applied during the early phases of biofilm development (Lawrence, Malone, Kober and Caldwell, 1989). Visualization of bacterial monolayers can be accomplished easily by either light (Duke and Michett, 1990; Herbert, 1990) or fluorescence microscopy (Jones and Simon, 1975; Sieracki, Johnson and Sieburth, 1985; Back and Kroll, 1991). Due to the resolution limits of optical microscopy, studies on thicker biofilms require either the use of expensive Confocal Laser Scanning Microscopes (CLSM) (Bloem, Veninga and Shepherd, 1995) or mechanical removal of the biofilms from the substratum prior to further analysis.

Image acquisition, processing and analysis techniques are well established in the biological sciences (Inoué, 1986; Russ, 1990) including microbial ecology (Dudley, du Plessis and Senior, 1994a). Thus, enumerations, determinations of cell size/morphology, attachment and microbial growth, and evaluation of responses to environmental stresses (Wynn-Williams, 1990) may all be examined. Improvements in methodology to estimate the number of microorganisms have resulted in the ability to generate data far more efficiently and with greater accuracy than previously possible (Treskatis, Orgeldinger and Wolf, 1997). The use of microscopy/image analysis to determine kinetic parameters of growth and to describe a sequence of events, such as biofilm colonization by a population of microorganisms, has evolved with the developed programmes.

Limitations to the more extensive use of fully automated image analysis in biology are essentially a lack of image contrast (measure of the variation in brightness between the lightest and darkest portions of an image) and the visually complex image (e.g. it might contain contiguous components or many dissimilar structures which possess a common grey-level, a fundamental element in the image analysis process).

1.5 *PRIMARY OBJECTIVES*

The principal objective of this research programme was to establish methodologies appropriate for definitive fundamental microecophysiology studies on the role of landfill covering soils (daily, intermediate and final) in leachate attenuation and subsequent landfill revegetation. Since soil particles provide surface area for microorganism attachment a second objective was to quantify and compare the concentrations of key leachate components catabolized by surface-attached and free-living microorganisms. This entailed an examination of primary adhesion as well as biofilm development on glass surfaces of undefined bacterial populations isolated from landfill. Development of sampling and microscopy techniques and image analysis hardware and software to determine the behaviour of both planktonic and surface-attached (biofilm-forming) microorganisms in microecophysiological terms was central to the study. The first step was to establish methods for studying the "real time" processes (of both individual microorganisms and associations) of attachment, establishment, division and growth on surfaces under constant and standardized conditions. The second step was to modify these methods to study microbial associations *in situ*.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 *ANALYSES*

2.1.1 Chemical

Hexanoic Acid: Hexanoic acid concentration determinations were made with a Varian 3600 gas chromatograph equipped with a flame ionization detector, Varian 8100 autosampler and an 80386 computer running a dedicated chromatograph programme, "Delta". The initial column temperature was 145°C which was held for five minutes and then increased to a final temperature of 160°C at a ramp rate of 3°C min^{-1}. The temperatures of the injector and detector were 210°C and 220°C, respectively and the flow rate of the nitrogen carrier gas was 25ml min^{-1}. Duplicate aqueous samples (1μl) were injected directly into the Langet (Durban, S.A.) stainless steel column (length 2m, i.d. 2mm) packed with W-AW support and FFAP/H_3PO_4 (10%.1%$^{-1}$) liquid phase. Concentrations were quantified by comparison of mean peak areas with suitable standards (Appendix One, p120). The standards (concentration range 0.5 - 50mM) were prepared once and stored in a freezer (-10°C) until use.

Catabolic Intermediates: Propionic, butyric and acetic acid concentrations were determined using the same system, programme and operating conditions as those for hexanoic acid. Duplicate aqueous samples (1μl) were used and were quantified by comparison of mean peak areas with suitable standards (0.5 - 50mM).

Culture Supernatant pH: pH values were determined with a CRISON Micro pH 2000 (Crison Instruments, Spain).

2.1.2 Microbiological

Dilution Plate Counts: To estimate the number of viable microbial cells, 1ml samples of culture supernatant were, if required, diluted with phosphate buffer (0.2M) and then inoculated into sterilized (121°C, 15min) cooled (48°C) basic mineral salts solution

(Watson-Craik, 1987) supplemented with hexanoic acid (10mM) and agar (2%m.v^{-1}). Individual colonies were counted after incubation (2d) at 30°C in a Controlled Environment Incubator (New Brunswick Scientific Co. Inc, Edison, N.J.).

Turbidity Measurements: Samples (5ml) were used for biomass estimations by measuring absorbance (wavelength 625nm) with a spectrophotometer (Milton Roy Spectronic 301).

2.1.3 Light Microscopy

An Olympus BH-2 (phase contrast) or a Zeiss Axiophot (differential interference contrast (DIC) and fluorescence) photomicroscope (Plate 2.1) were used. For image capture the microscopes were linked to video cameras via a microscope/TV adapter (MTV-3, Olympus for the JVC and HZ-M152 Victor for the Sony) to either of two video cameras: a JVC-KY-F30E colour video camera (Victor Company, Tokyo, Japan); and a Sony (Japan) CCD black and white camera, respectively. For fluorescence microscopy, the slides were prepared by standard methods (Trolldenier, 1973).

Free-Living Cell Visualization: To view free-living cells, culture samples (5ml) were first filtered (Millipore, 0.22μm). A negative staining technique, Nigrosin (1%m.v^{-1}) for one minute and then acridine orange (1:15000) for two minutes was used. A Zeiss Axiophot incident light fluorescence microscope with reflected light equipment (446440) was used with: a fluorescence illuminator and collector; a HBO 50-W mercury short-arc lamp (392642) with filter set 487901 (BP 365 exciter filter, FT 510 chromatic beam splitter, and LP 520 barrier filter); and Plan-Neofluar 20X (440040), 40X (440350) or 100X (440480) objectives.

2.1.4 Image Analysis

Image processing and analysis, and the development of image analysis methodology to quantitatively examine landfill microbial associations responsible for leachate catabolizm, are described in Chapter 3.

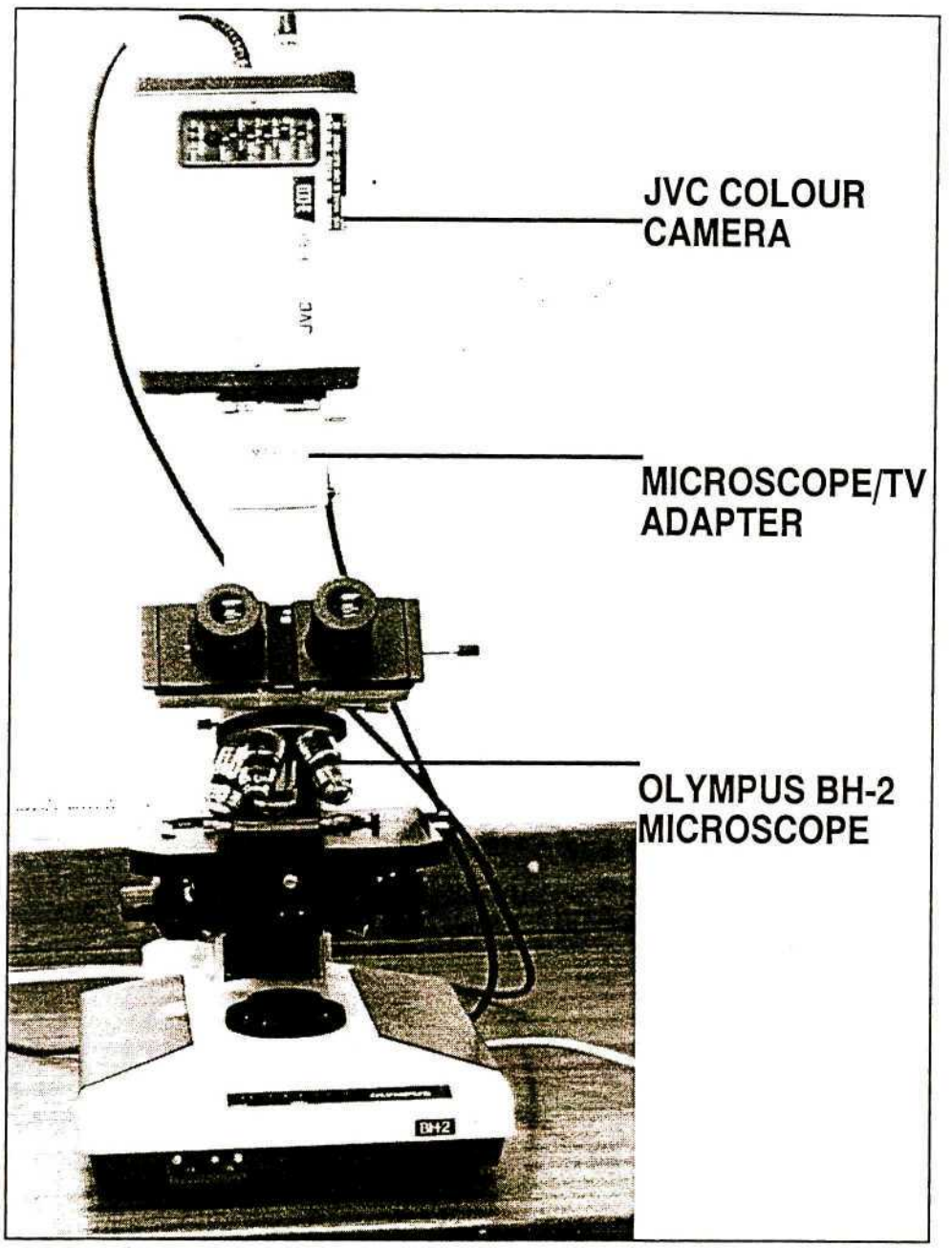


Plate 2.1. Image capture configuration consisting of microscope and camera. The camera was linked via a microscope/TV adapter (MTV-3, Olympus for the JVC camera (shown here) and adaptor HZ-M152 Victor for the Sony camera) to either an Olympus BH-2 (phase contrast) (shown here) or Zeiss Axiophot (differential interference contrast (DIC) and fluorescence) photomicroscope.

CHAPTER THREE

3. DEVELOPMENT OF IMAGE ANALYSIS METHODOLOGY TO QUANTITATIVELY EXAMINE LANDFILL MICROBIAL ASSOCIATIONS RESPONSIBLE FOR LEACHATE CATABOLIZM

3.1 *INTRODUCTION*

A useful technique for the quantification of microbial cells is image analysis (IA) (Jones, Watson-Craik and Senior, 1992b; Wynn-Williams, 1996) in which the quantification of digital images is carried out by making a large number of simple computations based on the use of pixels (picture elements). "Image analysis" consists of, firstly, image processing where an image is captured in analogue form by an electronic process, converted to digital form and then stored in an electronic memory. During this process the image may be modified in various ways to increase the contrast or enhance the information (Jarvis, 1988). Secondly, the processed image is analyzed by subjecting it to mathematical functions which extract a wide range of data from the captured image. "Image analysis" is used as a collective term for these two processes (Koeditz, 1986). A Kontron model Vidas 2.1 Digital Image Analyzer was used in this study. Terms specific to this system are explained in Appendix Two (p121).

Computer-based equipment allied to video digitizing techniques may be used to collect, process and display data for the purpose of extracting statistical information from a wide variety of source materials. The features of a modern IA system include:

image processing;

real time digitization (which converts the analogue information to digital form);

feature extraction (part of an image which can be isolated from the remainder by some means) and morphology recognition;

measurement of geometric, densitometric (grey level) and colour specific features;

functions for image processing and image analysis;

and measurement. By these means, biological microscopical images may be characterized and quantified in terms of their colour, optical density, shape and size or texture (Bradbury, 1983; Caldwell, 1985; Van Houtte *et al.*, 1995).

Image analysis is now a well established extension to optical microscopy, allowing routine quantification of microscopic observations (Inoué, 1986; Thomas and Paul, 1996). It is able to provide an accurate and rapid alternative to subjective human observation. Reproducibility, accuracy and speed are the key factors favouring the use of IA compared with visual observations (Häder, 1991).

The versatility of modern video equipment facilitates the collection and recording of images from a wide range of sources (Koeditz, 1986). A particularly versatile source of images for quantification is gained via high resolution video cameras interfaced directly with light microscopes. Thus, direct viewing, requiring transmitted or incident light as well as other image modes such as fluorescence or phase contrast, may be employed (Wynn-Williams, 1988; Häder, 1991; Walsby and Avery, 1996).

The more extensive use of fully automated IA in biology is usually limited by a lack of image contrast or by image complexity (similar grey-levels (brightness of each pixel in the image) or adjoining components) thereby making segmentation arduous. Despite these limitations, an increasing number of applications, including microbial adhesion; quantitation of microbial growth on surfaces and image analysis of anaerobic microbial biofilms on landfill material, have been developed in this field using modern apparatus, (Inoué, 1986).

3.1.1 **Image Requirements**

Sample preparation is extremely important for successful image analysis. Biological image analysis usually presents a greater challenge to the operator than many of the material science applications for which the equipment is more widely used. This is primarily due to the lack of contrast in the average biological specimen (Häder; 1991). To ensure accurate measurements of microbial growth (from microbial number, size increase or biofilm development), the images must be of the highest quality (Duke and Michett, 1990). The image to be analyzed requires sharp outlines for clear recognition of objects (Koeditz, 1986). Aggregated or superimposed particles require extensive processing to separate in order to obtain accurate results. Specimen preparation methods must, therefore, concentrate on rendering the highest possible contrast between the specimen and the background and thus allowing as clear as possible a distinction between seperate objects.

Image processing cannot selectively differentiate between objects as the human eye/brain can. It can only separate and quantify according to differences in either intensity, colour, size, shape or texture. Sample preparation facilitates segmentation (Koeditz, 1986). Effort spent in presenting the specimen in the best possible manner can prevent hours of complex electronic manipulation later. Most importantly, the presentation must be consistent from one sample to the next with special reference to lighting conditions (Bruton, 1994). An analytical sequence based upon grey-levels from an image is dependent upon the use of consistent methods. The microscope magnification, optical distortion and camera enlargement used will determine the level of detail observed and will similarly affect the absolute values for all parameters used (Duke and Michett, 1990). Only if such conditions are consistent for all samples can legitimate comparisons be made.

A vital aspect of sample preparation is the exclusion of all extraneous matter which will not form part of the desired image. The larger the screen image of the subject of interest the more accurate the results will be. For good estimates of parameters, the particles require a surface area of at least 100 pixels (due to edge effects). The operator must select the appropriate equipment amongst those locally available - video camera with macro lens (black and white or colour), stereo microscope or compound microscope - for the task to be undertaken. The 'ideal' image for image analysis should fulfil the requirements below: (Bruton, 1994)

Small size variations in the objects (Geometric resolution);

High contrast (Densitometric (grey-level) resolution);

No geometric distortions;

No agglomerated structures (Object separation);

Good signal/noise ratio;

Sharp edges and contours to the objects;

Homogenous background (shading).

These ideal conditions seldom occur in practice. Modern apparatus has, however, a wide range of image preprocessing and postprocessing functions (see section 3.3) to render all but the most impossible specimens suitable for analysis (Nivens, Palmer and White, 1995).

3.1.2 Segmentation

Segmentation is the process of dividing a digital image into distinct regions by defining each individual picture element (pixel) (Sieracki, Reichenback and Webb, 1989). An advanced IA system is capable of discriminating objects (the process of separating an image into the various features and separating those parts of an image which are of interest from those which are not (the "background")) on the basis of five different parameters, i.e. size, shape, illumination intensity, colour and texture. The simpler (more "ideal") the image is the easier it becomes to perform accurate, consistent segmentation. If satisfactory segmentation of the objects cannot be carried out at the first attempt, then alternative image preprocessing and postprocessing functions may be used to improve the accuracy of the segmentation (Busscher and Van Der Mei, 1995).

Interactive thresholding (compared to automatic) can be used to define features. The technique is effective and the resulting binary image allows simple measurement. Visual thresholding (the alternative) requires time-consuming human intervention, even if it is made globally (i.e. one threshold for an entire image) (Sieracki *et al.*, 1989). Local thresholds (i.e. one threshold for each object) permit more accurate measurements but are excessively time consuming (Jarvis, 1988). Extreme subjectivity can, however, be introduced with "human" thresholding. The lookup table (LUT) design of modern video digitizing hardware is ideally suited to the implementation of thresholding and image editing. A LUT is a means whereby the digitized video signal may be altered to allow one to improve the image for viewing purposes without affecting the integrity of your data for analytical purposes. Thresholding converts a grey-level image to a binary image by comparing each pixel value to a threshold value. Highlighting differences in the light intensity causes the edges of the object to be clearly distinguished from the background allowing segmentation to be more effortlessly achieved (Russ, 1990).

3.1.3 Measurement

Having obtained an image of high quality, the measurement of areas, perimeters and density is easy and rapid with automated systems (Wynn-Williams, 1996). Consequently, many such measures may be combined into multivariate plots in an effort to obtain a synopsis (Posch, Pernthaler, Alfreider and Psenner, 1997). This could serve to diagnose,

for example, the concentration at which leachate is inhibitory to surface-attached cells (Dudley, Wallis, Bruton and Senior, 1992b). Direct measurements of sizes or of calculated shape descriptors may be used with upper and lower boundaries as "windows" for the exclusion of unwanted features from the measurements (Caldwell, 1985). Appendix Three (p125) contains a full list of the measurements used in the present study. Ideally, if a shape factor is used it must be sensitive to small changes in shape, be independent of any other properties of the feature, be dimensionless and rotation invariat (Bradbury, 1983).

3.1.4 **Microdensitometry**

One of the most useful measurements available from a TV-based system is the integrated optical density of a feature or field (Jarvis, 1981). Integrated optical density represents the total image "brightness" and is often referred to as the "mass" of the image. Microdensitometry involves measurement of the absorption of light by microscopic specimens usually to determine the integrated optical density which is proportional to the mass of the absorbing substance (Jarvis, 1981). This can give valuable information which can be used to discriminate numerically between visually similar textures (Caldwell, 1985). Such integrated density measurements applied to a series of features (eg. cell density) can be used to produce histograms of the distribution of densities against, for example, colony area. Such histograms may be used, for example, in the study of chemical toxicity (Dudley, Howgrave-Graham, Bruton and Wallis, 1993a). Using the configuration described, grey-level readings, intensity values and optical density or transmission values can be used to monitor microbial replication and biofilm development.

3.2 *MATERIALS AND METHODS*

3.2.1 Hardware

The Centre for Electron Microscopy of the University of Natal, Pietermaritzburg has a Kontron Vidas 2.1 Digital Image Analyzer (Plate 3.1).

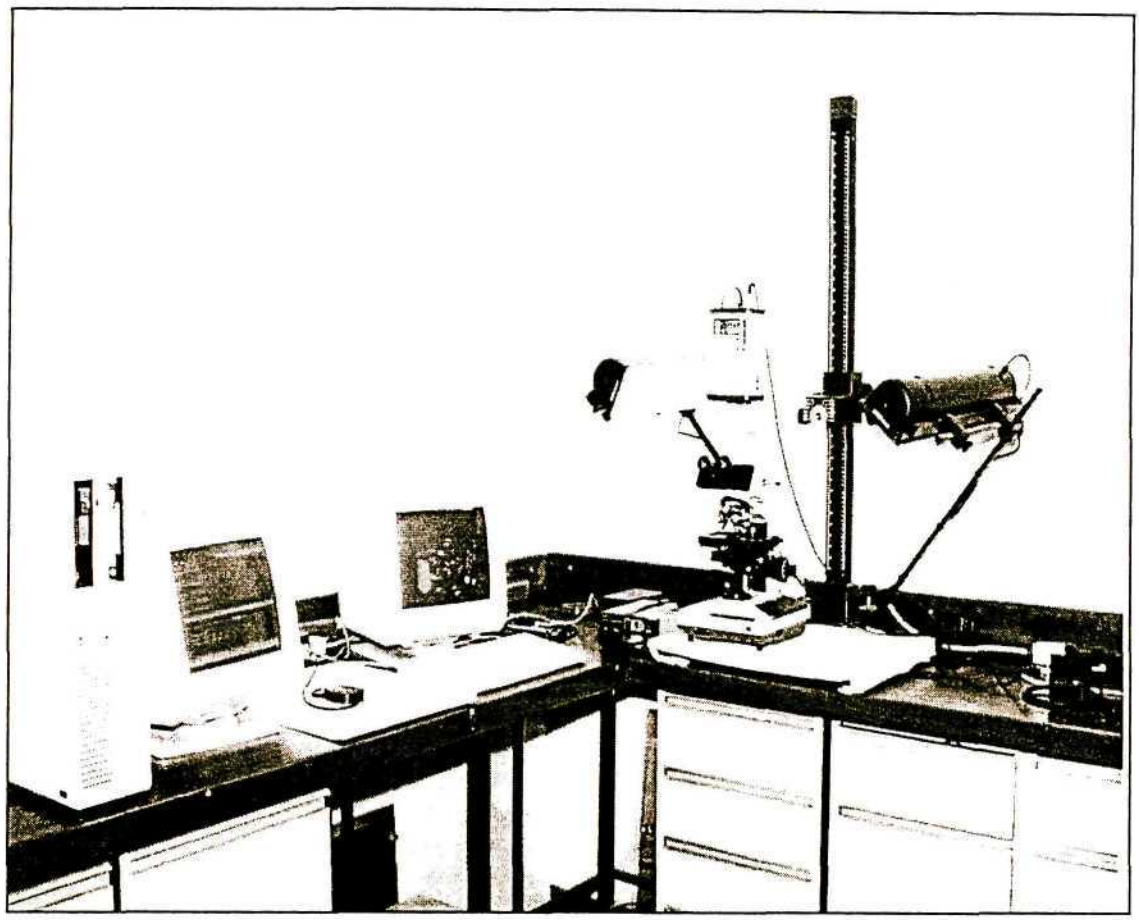


Plate 3.1. Kontron Vidas 2.1 Video Image Analysis system for analyzing images of biofilm development.

The Vidas system consists of the following hardware components: central processor unit (CPU); video frame grabber and storage unit; VGA monitor (menu display); RGB monitor (image display); keyboard; digitizer tablet (Kontron Elektronik, 1991) and cross-hair cursor (Digicad); TV camera (Sony CCD B/W or JVC CCD RGB); and laser printer (Plate 3.1). The CPU is based on an 80386/80387 processor, operating at 20MHz clock rate. The unit

contains two free serial interfaces (RS 232) and one parallel Centronics compatible interface. A black and white image measuring a standard 512 x 512 pixels requires 0.36 megabytes of storage capacity. The system described used Bernoulli (IOMEGA, USA) removable cartridges of 44 megabyte capacity for image storage.

3.2.2 **Software**

Although hardware development is fundamental to high-quality capture of video images, the heart of any image analysis system lies in the software. It is the software which dictates the speed, capacity and versatility of the apparatus. Modern imaging equipment is provided with software libraries (Jarvis, 1988) which relieve the burden of low-level development (Kontron Elektronik, 1991) although the latter is still no trivial task. Many recent designs now feature user-friendly menu-driven layouts where very little skilled input is required from the operator. Other designs, usually of extended capacity, require input where low-level programming skills become essential (Jarvis, 1988). The Kontron Vidas system used in this study requires such programming skills.

3.2.3 **Macros**

Macros comprise a series of simple computer commands which are linked together in a particular sequence to enable the IA computer to perform a series of specific IA functions (Figure 3.1). The use of macros is essential to structure complex sequences efficiently. Macros may be "self standing" or they may have human intervention steps built into them.

Major Functional Steps in Image Analysis: Listed below are the major functional steps of an image analysis process (Koeditz, 1986) (Figure 3.1).

CALIBRATION (see section 3.3.5.ii) - Allows image scale setting either interactively and/or automatically at the required magnification.

IMAGE DIGITIZATION AND FRAME STORING (see section 3.3.4 & 5.iv/v) - Capturing the image via a camera mounted on a copy stand or microscope and storing this image in digital form.

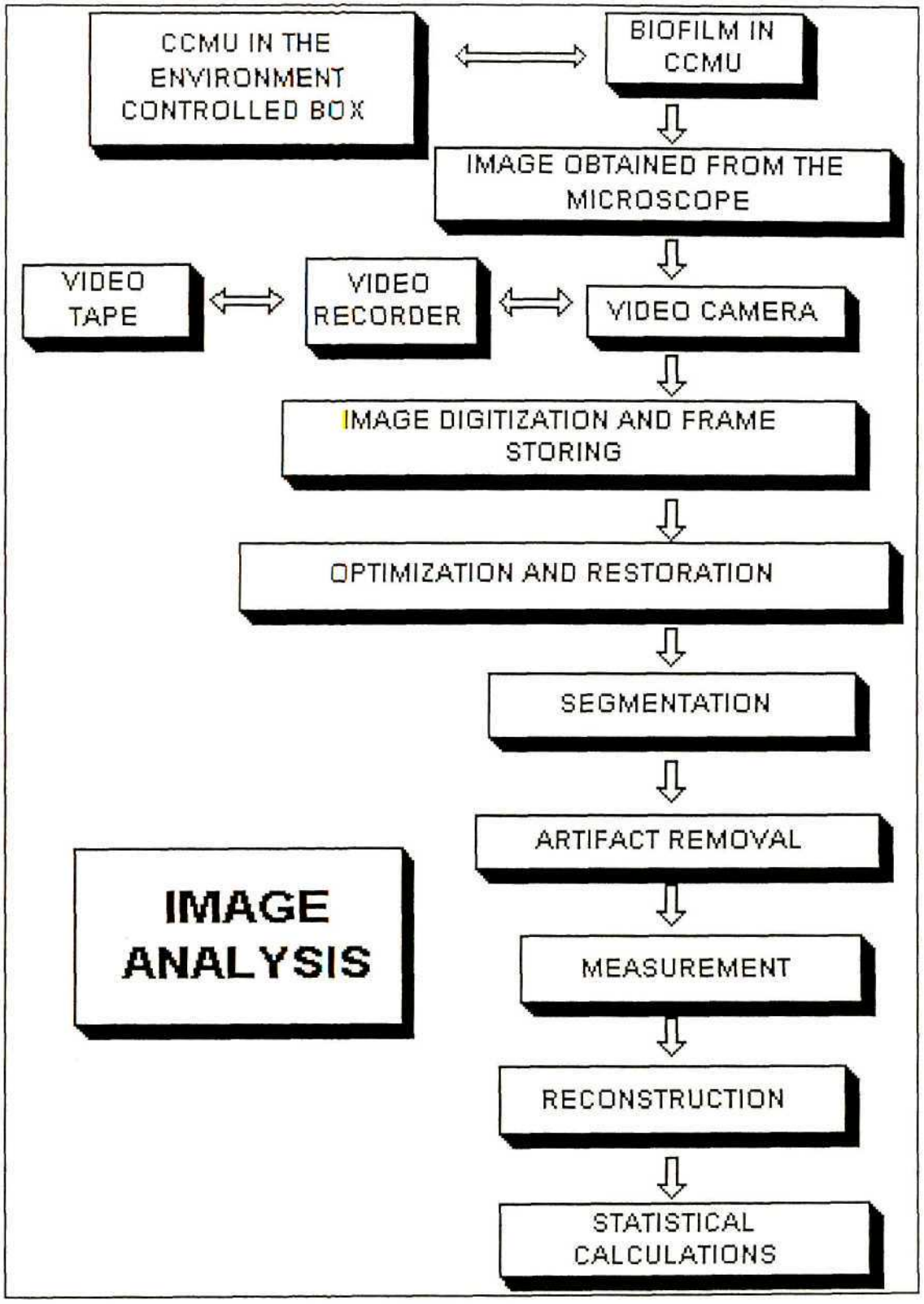


Figure 3.1. Flow diagram of the method of biofilm analysis with an image analyzer.

IMAGE OPTIMIZATION AND RESTORATION (see section 3.3.5.vi/vii/viii) - Steps to optimize the image in its captured form, e.g. enhancing contrast, edges and a variety of other parameters. The aim is to optimize the image for further processing.

SEGMENTATION (see section 3.3.5.x) - Using grey-levels to separate the image into different segments which may then be quantified. Segmentation is the process that subdivides an image into its designated constituent parts or objects. This step is extremely important as it forms the basis for all further image processing and analysis. Segmentation may be performed on the basis of: size, shape, grey-level, colour or texture.

ARTIFACT REMOVAL (see section 3.3.5.xi) - Identifying and removing irrelevant or invalid information from the collected image.

MEASUREMENT (see section 3.3.5.xiii) - Making counts at pixel level on the different objects previously identified e.g. number, length of cell, area of object and optical density of object.

RECONSTRUCTION (see section 3.3.5.xiv) - Overlaying the counted areas over the original image for verification.

STATISTICAL CALCULATIONS - Performing calculations on the data which have been gathered.

IMAGE ARCHIVING - Storing the digital image in an electronic storage device or cartridge.

IMAGE DOCUMENTATION / REPORT WRITING - Presenting the image and statistical information (including histograms and data lists) in the form of a report.

NETWORKING - Networking to other systems if desired.

Three Main Image Analysis Modes: Depending on the nature and extent of the application there are three different modes of IA which can be used. These are: semi-automatic, fully automatic and interactive IA (Koeditz, 1986).

Semi-automatic: Where specific applications are not labour intensive, are temporally short or where structures are so complex that preparation for automatic analysis is not feasible (Van Houtte *et al.*, 1995) then semi-automatic IA may be employed (eg. Kontron Videoplan). With such computer-assisted but manual input systems, the operator traces the identified contour on the digitizer tablet with a cross-hair cursor. The computer then calculates the required geometric parameters automatically.

Fully automatic: For faster results, fully automatic IA, incurring the writing of macros, is desirable (Moller *et al.*, 1995). A sensible precaution is to ensure that the programming time does not exceed the time it would have taken to complete the task semi-automatically. Although the automated procedure reduces the measurement time per specimen dramatically, a prerequisite is that it must be feasible to extract the structures to be measured. This is largely dependent on specimen contrast. If segmentation is difficult then the reproducibility is diminished.

Interactive: A macro which runs automatically, but also requires manual inputs, is referred to as interactive IA. This technique was used in this project, for example, for the quantification of fluorescent bacteria on surfaces (light micrographs of surfaces using an epifluorescence microscope), and the accurate determination of the growth rate of morphologically distinguished microorganisms involved in the bioremediation of an oil-contaminated site (intermittent live image capture from a phase contrast microscope) (Dudley *et al.*, 1992a). Demonstration macro three (see section 3.3.6.iii and Appendix Five, p133) is an example of such a macro as used in this study.

The following features are specific to the Kontron Image Analyzer as used in this study and are provided as an example of typical steps in the image analysis process.

Writing a Simple Macro: Macros in the Kontron Vidas IA all contain (cycle through) similar basic steps. The specific commands which follow are dependent on the nature of the image and the desired result. The macro used as an example (below) was written to count

the number and individual area of bacterial cells in a sample. The basic steps followed (and necessary in every measurement sequence) were: initialization, calibration, image capture, segmentation, identification, measurement and data output. The words in bold type are the **FUNCTIONS**, followed by an explanation of what that function does.

RESETPAR	→	resets all parameters
MEASSTOP	→	terminates all previous measurements
CLEARALL	→	clears all images
SETFRAME "F512"	→	sets format of image to be processed
LOADLUT "grey"	→	Selects the LUT (what colours used)
TV CHAN 2,2	→	adjusts the system to different input channels + input modes. The display mode is set to colour
TVON	→	Switches the system to external synchronization and displays a live TV image on the monitor
TVINP ONE	→	Digitizes a single TV cycle and stores image ONE If colour was used then the TVINP are: ONE = RED; FIVE = BLUE; NINE = GREEN
TV CHAN 0,0	→	Changes the TV channel back to black and white
DIS TWO LEV	→	This discriminates objects from the background by setting upper and lower thresholds. This can be done numerically or interactively
IDENTIFY	→	Connects pixels in a binary image. Every object is assigned its own grey-level and a LUT displays neighbouring objects in contrasting colours
INIT OBJ	→	This is used to declare object-specific parameters for measurement
MEASOBJ	→	Extracts the object-specific parameters (previously defined by function Init Obj) from an identified image and stores data in a file
OUTLIST	→	This displays the data measured as a list
OUTHIST	→	This displays the distribution of a selected parameter in a data file as a histogram.

3.2.4 Software Development and Testing with Particular Reference to Specific Problems/Limitations Associated with Landfill Microbiology

i. Objectives

One of the objectives of this project was to determine if the use of an image analyzer allowed direct measurement of, essentially, independent processes contributing to the colonization of substrata. The aim of the image analysis component of the study was to determine if IA could be used as a diagnostic tool for landfill soil-microbial interactions.

Specifically, this project was designed to determine if IA could be used to calculate specimen number, area, grey-level intensity and other required enumerations of landfill microbial species *in situ*. To do this, it was necessary to develop the required software (see section 3.3.2 - the numerous macro routines required for analysis of the biofilm). Different combinations of macros, hardware and image storage devices were compared for efficiency and reproducibility of the results.

ii. Function Specification

The fundamental specifications of a light microscope biofilm analysis system are relatively simple. A clear definition of the required function of the IA system is necessary for both hardware selection and software design (Jarvis, 1988). The system must be capable of detecting and segmenting image features from a composite video image. Different types of images to be analyzed also influence software design and hardware selection at an early stage. The assumption can be made that the features to be analyzed will not be easily discriminated from their surroundings. This may be due to the complexity of biological images and either insufficiently resolved light microscope images or low power fluorescence intensity. The system must allow for the exclusion of unwanted components or the inclusion of components not selected. Fast, automatic and accurate geometric and densitometric measurements must be made on the defined image features.

iii. System Hardware

The Kontron Vidas 2.1 IA system is based on a 80386/80387 host computer, running under MS-DOS 6.0 (Microsoft (R) Corp.) (Plate 3.1). The video signal was converted to digital form, at a frame rate of 1/40s, by the array processor which contained four colour and twelve black and white direct access image memories. The resolution of the digitizer was 768 X 512 pixels with eight bits of memory available for each of the three colours. This is equivalent to 256 possible grey-levels for each pixel. The system produced a high-resolution digitized colour image which was visually indistinguishable from a direct video image on a 27cm monitor. Each image was analyzed by the dedicated 80386 processor using the on-board library and imaging commands (Kontron Elektronik, 1991). The array processor was controlled by a host computer connected to a hard disk for programme and data storage. Instructions to the host computer were given via a keyboard or digitizer tablet with a cross-hair cursor. The latter provided the input for simple interactive image editing. It was possible to view the image resulting from each step of the image processing on a monitor and to compare it with the original video image. A second monitor displayed the macro in use.

A printer produced an output of the measured data. A series of Bernoulli (IOMEGA, USA) removable cartidges of 44 megabyte capacity each were used for image storage. Using double space (MS-DOS 6.0), over 75 megabytes were available for image storage on each disk. The average size of each grey-level image was 360 kilobytes. A standard video recorder (P7, JVC) stored the images on video tape (BASF, E30 HG). It was determined that 15s were sufficient for multiple image capture (1/40s image^{-1}) and the same time interval was used to record the image details. Figure 3.1 is a flow diagram showing the manner in which the images were captured and analyzed.

3.3 *RESULTS*

3.3.1 **Image Analysis for Biofilm Investigation**

The ecology of a biofilm is a complex function which includes prevailing growth conditions, hydrodynamic forces and the dominant microbial inhabitants in the biofilm (Lappin-Scott *et al.*, 1993). If the biofilm is disrupted, essential information is lost (Fletcher, 1984). The

organization of biofilms must be understood to more accurately identify growth rate-limiting events and diffusion coefficients for a host of biofilm processes (Breznak, 1984; Lorenz, Aardema and Krumbein, 1988; Marshall, 1988; Kober, Lawrence, Hendry and Caldwell, 1992). Nine cellulolytic bacterial isolates obtained from landfilled refuse were easily differentiated on the basis of gross morphological characteristics by Westlake (*et al.*, 1995) showing the potential uses of the image analysis morphology differentiation system.

The variations in environmental conditions and concentrations of substances which might affect the microbial biofilms must be determined if the landfill bioreactor is to be understood and successfully managed. The disruption or destruction of the biofilm during analysis can be avoided or minimized by use of image analysis (Dudley, 1993).

3.3.2 **Software**

Software (in the form of ready-to-use macros) was not available for the planned application and had to be written. Control programmes were written in the Vidas (Kontron) environment, using standard built-in commands, to cycle through a number of captured image inputs (Kontron Elektronik, 1991). These programmes were designed to automate the processing, analysis and measurement procedures and to log the results for subsequent analysis. The different IA steps were separated into macros (Table 3.1).

These macros were linked to form the diverse programmes used (Table 3.2). The full software is detailed in Appendices Three (p125) and Four (p130) and is lodged in the Macro Library of the Centre for Electron Microscopy, University of Natal (E-Mail: Bruton@emu.unp.ac.za). Due to space and time constraints the images were analyzed in groups of 100 with each step of the programme made on each image. The computer-intensive steps were done overnight while the interactive steps were performed simultaneously to minimize operator time. The stand alone programmes are listed in Appendix Four (p130).

Table 3.1. Macros constructed with a Kontron Vidas 2.1 Image Analyzer to quantify surface-attached biofilm development. The list is the fundamental strategy in which the different macros were combined in the programmes to achieve the desired result.

MACRO NAME	FUNCTION OF THE MACRO
-setup	Complete system initialization
-pset	Partial system initialization
-scale_1	Scale setting, interactive or from memory
-scale_2	Calibrated scales
-medbp_1	Measurement and data base parameter setting for two sets of data bases (five data bases in each set)
-medbp_2	Either parameter initialization or opening of relevant data bases
-imcap	Image capture and addition from video recorder
-bgcor	Background correction
-imsr_1	Single image storage or retrieval
-imsr_2	Multiple image retrieval and storage
-hispr	Histograms, probability of each pixel value
-filtr	Image enhancement with different filters
-glppd	Grey-level image processing
-biimp	Binary image processing
-seg_1	Single image with multiple phase segmentation or at two threshold levels
-seg_2	Multiple image segmentation (two threshold levels) with checks and correction
-zoom	Zoom, up/down
-indent	Identification, with selection of frame size
-dbinf	Data base information setting
-measf	Measurement of field based, object specific and densitometric parameters
-smdbp_1	Opening of data bases, measurement of objects and storage into different data bases with classification shown on image screen
-smdbp_2	As in -smdbp_1 but with six images simultaneously
-resul_1	Results as a list, display or printing
-resul_2	Results as histograms, scattergrams, distrograms, cross or prob.
-resul_3	Results displayed on the image screen
-deldb	Data base deletion

Table 3.2. **Stand alone programmes for a Kontron Vidas 2.1 Image Analyzer used to quantify surface-attached microbial growth.**

NAME	FUNCTION
Background correction	Multiple image retrieval, background correction and storage of new images
Automatic processing	Large-scale automatic processing of images
Image enhancement	Large-scale addition, retrieval and storage of images
Segmentation	Large-scale segmentation of many images
Measurement macro 1	Automatic measurement of segmented images
Measurement macro 2	Used to selectively measure microorganisms in segmented images
Measurement macro 3	Measurement of large numbers of segmented images
Reproducibility	Used to check the reproducibility of the image analyzer

Table 3.3. **Demonstration programmes which show how different complexities of images of growing microorganisms can be quantified by image analysis.**

NAME	DESCRIPTION
Demonstration macro 1	Basic procedure to analyze an image. Uses stored images to demonstrate how area determinations are achieved.
Demonstration macro 2	Demonstration of the basic procedure to analyze a large number of images
Demonstration macro 3	Macro used to analyze multiple images with different magnification

The demonstration programmes (Table 3.3) show how different types of biofilms may be quantified by IA. Details of the demonstration programmes are presented in Appendix Five (p133). A number of image analysis terms specific to the Kontron system are used in this thesis and these are explained in Appendix Two (p121).

3.3.3 **Reproducibility**

The reproducibility of the image analysis process was tested as follows (Bolton, Morris and Boddy, 1991) (Table 3.4):

Test One. The video camera, microscope and image (of cells on surfaces in a CCMU) were positioned for image capture and the macro (-imcap) was run approximately 100 times with the same image. All image manipulations were made automatically for each sequence of the macro, i.e. the result reflected the programme only;

Test Two. The system was set up and run as for test one but segmentation was effected interactively to determine the effect of interactive discrimination (macro - imsr_1);

Test Three. The video camera, microscope and image were positioned for image capture on video. Numerous images of the same field were captured. Each image was analyzed independently by the macro. All image manipulations were made automatically for each sequence of the macro to determine the effect of capturing images from a video recorder (macro - imsr_2); and

Test Four. The system was set up and run as described in test three but the CCMU was removed and replaced (imitating normal experimental procedure) before image capture of the same field to determine the effect of returning to the same field of view (macro - imsr_1).

From Table 3.4 it is clear that the actual IA programme did not introduce any errors while the cumulative effect did not introduce significant errors into the results. The IA test which introduced the highest variation was segmentation (0.0248) but this was not judged to be significant. The use of video tapes to store the images introduced a small error (0.1541) which was not judged to be significant.

Table 3.4. Reproducibility of Kontron Vidas 2.1 Image Analyzer used to measure images of microorganisms as AREAP (Relative area of the discriminated phase).

TEST	No. OF BACTERIA	No. OF FIELDS	MEAN	AREA	VARIATION	STANDARD DEVIATION
1. - Effect of the programme	2652	78	1.146	89.440	0	0
2. - Interactive discrimination	2999	89	1.218	108.41	0.0006	0.0248
3. - Capturing images from video	3230	101	1.889	190.84	0.0238	0.1541
4. - Removal/ replacment and accumulative effects of above	3595	100	0.734	73.480	0.0508	0.2253

3.3.4 Image Storage

Digitization of the biofilms, with an on-line television camera and image processor, was not the best method due to the high cost of storing digitized images on disk and disk errors occasionally resulted in the loss of images. Images were, therefore, recorded on video tape to provide an inexpensive hard copy of raw image data. This procedure isolated computer breakdowns and other processing difficulties from the CCMU experiments. Video capture also reduced the length of time which the cells were exposed to microscope illumination during collection of image data. The image of each colony can be digitized many times and the images can be summed to reduce electronic noise originating within the television camera.

3.3.5 Control Programme

Macros were written in the Vidas environment using standard built-in commands to cycle through a number of captured image inputs. These programmes were designed to automate the procedure and to log results for subsequent analysis. The different IA steps were separated into macros (Table 3.1) which were linked to form the programmes used

(Table 3.2). The macros are described below in the order in which they were used to analyze images. Depending on the image quality, steps in the macros may be selected or excluded from the programme in order to modify the image to facilitate image analysis. Detailed descriptions of the macros are presented in Appendix Three (p125). The main points of each of the macros are summarized below.

i. Initialization

The macro "-setup" is run at the start of any programme (Appendix Three, p128). This ensures that the IA is in the same format each time it is used. Previous images and overlays are removed, any measurement parameters which were active are stopped, all parameters are reset, the graphics display is set to alphanumeric mode, the image acquisition path is set to F drive (Bernoulli disk), the frame is set to maximum (768 x 512 pixels), a LUT is loaded (grey), the overlay colour is set (green), the initial display is set to one, the television channel is set to black and white, synchronization is set to composite signal, all messages in the message screen are erased and the necessary text and numerical values are initiated. The macro "-pset" is used to initiate the required parameters between individual images for group analysis (Appendix Three, p127).

ii. Calibration Functions

The calibration macros allow image scale setting, both interactively (macro "-scale_1") and automatically (macro "-scale_2") (Appendix Three, p127). All necessary scales are entered before analysis is undertaken. Images, at the required magnification, of an Objective Micrometer (Swift, Japan, category MA663, no. 10) are captured on both the X and Y axes (plane) as the pixels of the camera are not square, thus forcing calibration in both dimensions (Figures 3.2a and b). The two complementary images at the same magnification are retrieved and a normalized addition produces the image used for the setting of the measurement (Figure 3.2c). The desired magnification is selected depending on the magnification used on the microscope and the number of cells to be analysed (Figure 3.2d) and shown as a grid on the screen to check the scale. Each new scale measurement is then stored. Once this is completed the operator selects from the list, for any set of images, the scale required.

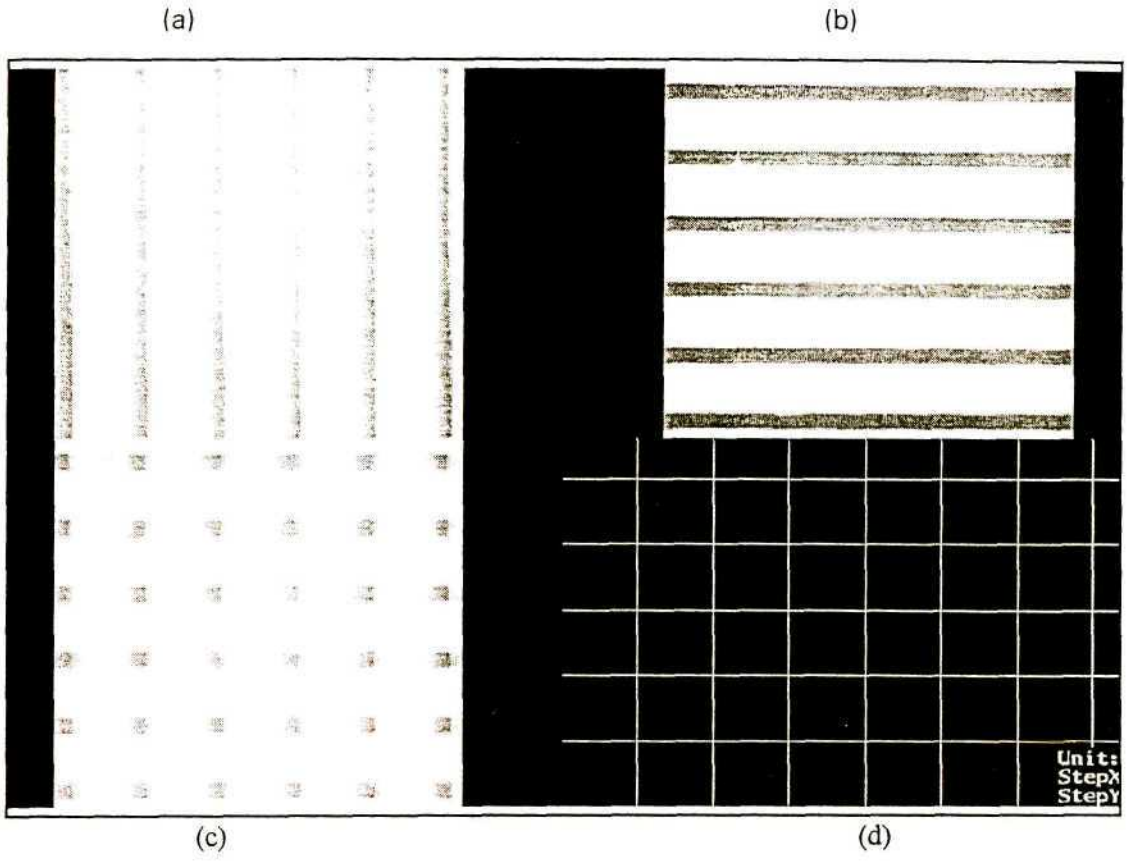


Figure 3.2. **Calibration, scale setting and image check for a Zeiss Axiophot microscope captured on a Kontron Vidas 2.1 Image Analyzer. a. Image CZ6B5X of a micrometre in the X direction. b. Image CZ6B5Y at the same magnification in the Y direction. c. Addition of images in X and Y directions. d. Image of the overlay from the scale setting image used to check the scale.**

iii. Measurement and Data Base Parameters

The macros "-medbp_1" and "-medbp_2" assign data bases for the collection of logged results (Appendix Three, pp127 & 128). Two sets of data bases are initialized, the first for the results of each image analyzed and the second for the overall results of the continuation, as the image from which the results come needs to be specified. Of a possible 34 different measurements three objective based measurements (area (Equation 3.1), fcircle (circularity) (Equation 3.2) and elongation (length = largest Feret diameter.shortest Feret diameter^{-1}) (Equation 3.3)) and three field based measurements

(area, relative area and field count) were used in this study after appraisal of the possible measurements. Each data base is erased, the feature vectors are specified and the data base is created and opened.

$$\text{AREA} = \text{NUMBER OF PIXELS X SCALED PIXEL AREA} \qquad (3.1)$$

$$\text{FCIRCLE} = \text{PERIMETER}^2.(4.\pi.\text{AREA})^{-1} \qquad (3.2)$$

$$\text{ELONG} = \text{DMAX.DMIN}^{-1} \qquad (3.3)$$

iv. Image Capture

The detrimental effect (narrow range of grey-levels) of the image stored on video is countered by the beneficial effect of image addition (increases the range of available grey-levels). While the quality may not be as high as digitizing a live image, the software routine ensures that the information loss is minimized. In the macro "-imcap" each image is digitized six times and additions performed (Appendix Three, p126). Image one is added to image two. This image is then added to another summed image, and so on, until each of the six images is added together. The separation of grey-levels into entities, essential for segmentation, may then occur with greater sensitivity.

If the addition of images does not improve the image quality, a number of other mathematical combinations may be used. Examples include multiplication or division, combination, minimum or maximum, and addition or subtraction. The image characteristics and what one wants to improve, or remove, determines which mathematical combinations should be used.

v. Image Storage and Retrieval

Where possible, groups of images are retrieved (macro "-imsr_2") and used but when complex or problem images are evident individual images are used (macro "-imsr_1") (Appendix Three, p126). The initial image storage from video to Bernoulli disk is always in automatic mode with multiple image capture and storage.

vi. Histogram

A simple visual observation of the image is often insufficient to determine if an IA procedure is advantageous or detrimental to the image. Use of the macro "-hispr" enables a more calculated selection of the correct procedure to analyze the image (Figure 3.3) (Appendix Three, p126). By comparing the width, height and number of peaks evident in the histogram (distribution showing the number of pixels which have a particular intensity value), the different influences of image processing on the image can be objectively evaluated (Figure 3.3).

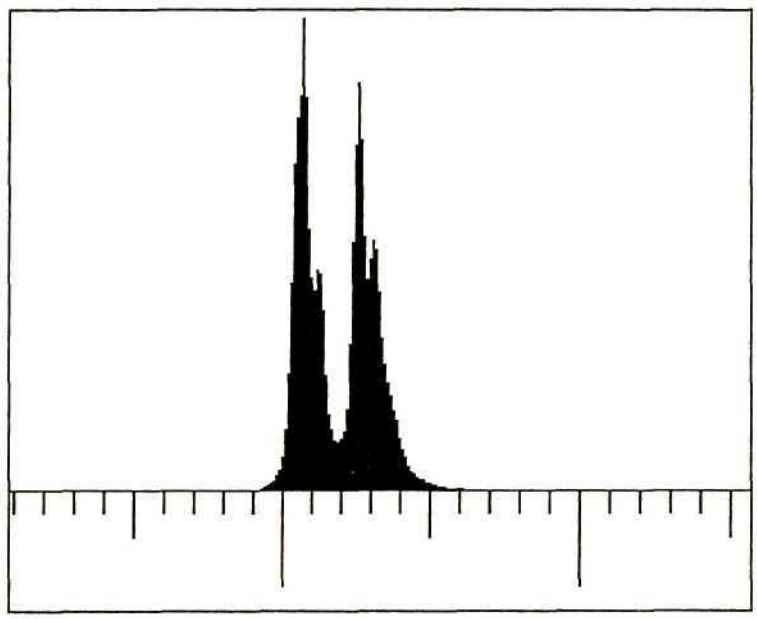


Figure 3.3. Grey-level histogram. By comparing the width, height and number of peaks evident, the effect of image processing on the image can be objectively evaluated.

vii. Background Correction

To ensure accurate and consistent segmentation, faults in the illumination system of the microscope must be corrected (Figure 3.4). The microscope may not be perfectly aligned each time. This will cause changes in the background illumination of different images. Background correction by the macro "-bgcor" is done in two ways (Appendix Three, p125).

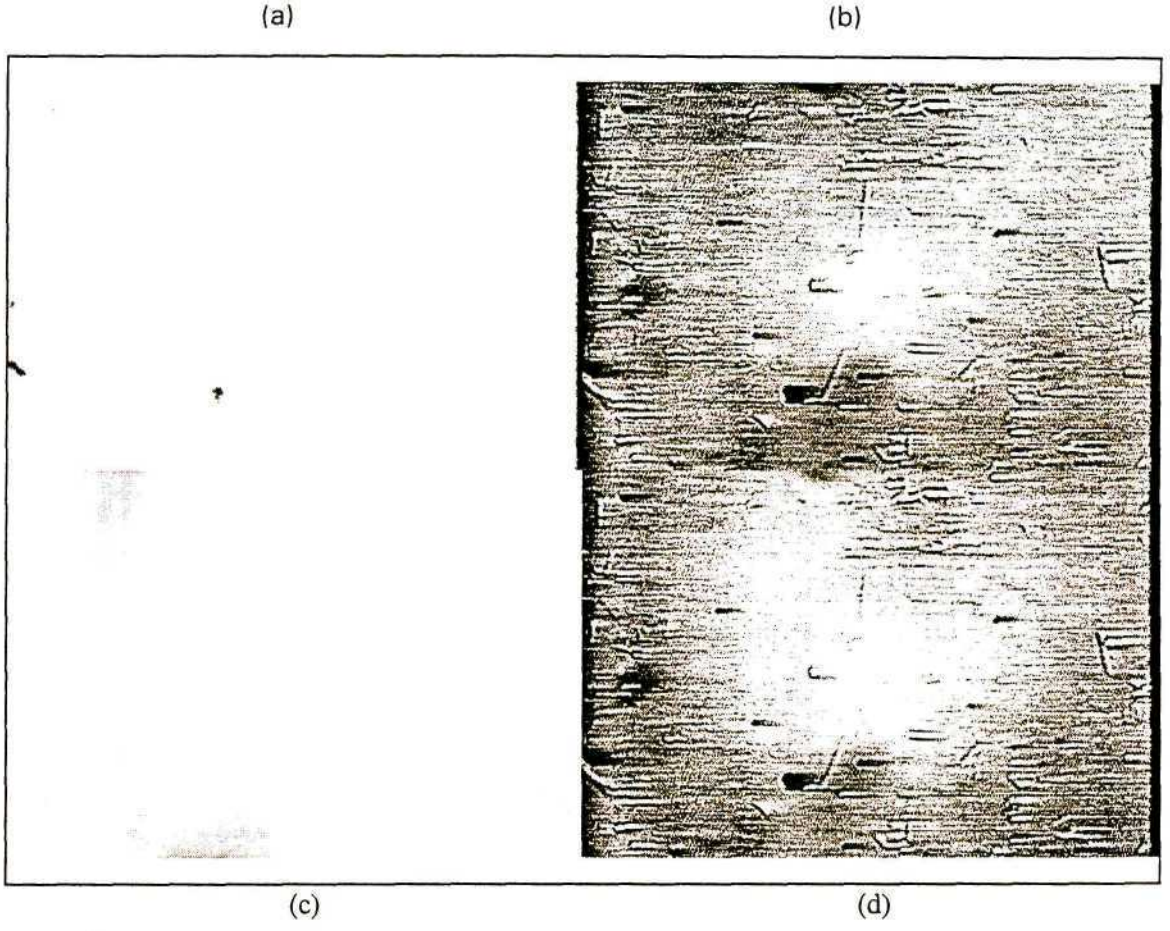


Figure 3.4. Example of the process of background correction in an image from a Kontron Vidas 2.1 Image Analyzer. a. Original image of bacterial growth. b. Linearized histogram filter. c. Background image used to correct image. d. Background corrected image.

Contamination in the light path or other shading variations of the illumination in the microscope are corrected by subtraction of a reference image from every image to be

analyzed. This is accomplished by selecting a clear field of view, defocusing the microscope at the required magnification and capturing the resultant image. This corrected image is then used to generate an illumination-corrected image by tracking the background (Figure 3.4c) and changing the default depending on whether it is a fluorescence image (light on dark background) or a DIC/phase contrast image (dark on light). This image is then used to create a shade corrected image (Figure 3.4d).

viii. Filter Enhancement

The various filters (programme algorithms which separate signals) available were evaluated and the most appropriate were used for each application by selection of the grey-level histogram produced (Figure 3.3). The four broad types of filters available with the Kontron Vidas 2.1 Image Analyzer were: 1 - contrast; 2 - smoothing; 3 - sharpening and; 4 - edges at different degrees; matrix sizes; threshold values; and factors or grey-levels.

1 - Contrast group of filters: normalize, linearize, scale grey-level, interscaling, transform grey and invert.

2 - Smoothing group of filters: low pass, sigma, median, rank operation, non-linear low pass, weighted lowpass and Gaussian.

3 - Sharpening group of filters: delineate, emphasize and enhance contour.

4 - Edges group of filters: Sobel gradient, Roberts gradient, sum gradient, max gradient, high pass and Laplacian.

Depending on the particular characteristics or source of the image to be analysed, one or a combination of the above were used. Figure 3.5 shows how different filters were used to selectively improve different aspects of various images and Figure 3.6 represents the resultant histogram. In fluorescence lighting a contour enhancement filter was used to provide a selective enhancement of well-demarcated objects by sharpening the edges. For DIC, a normalize filter (factor one) and then a contour enhancement filter (matrix size three, degree two) were used.

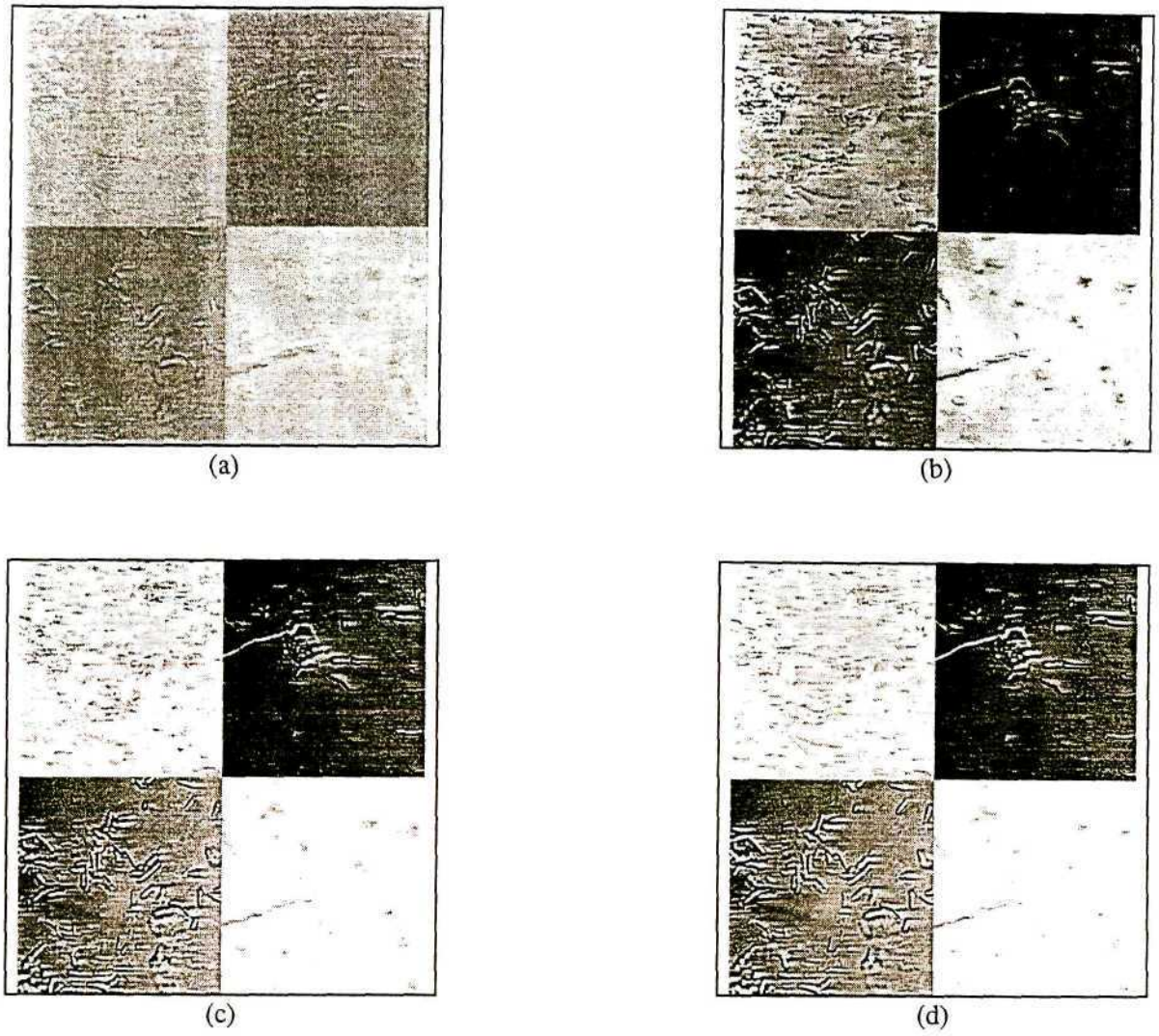


Figure 3.5. **Image of growing landfill microbial association captured with a Kontron Vidas 2.1 Image Analyzer from original image to filter enhancement. a. Original image. b. Filter enhanced (normalized). c. Background corrected. d. Filter enhanced (using the filter "enhance contour").**

ix. Zoom

For many images it is useful to be able to zoom into a particular area to enlarge the captured image or to reduce the image size to combine four images into one. Zooming has obvious advantages for microorganism recognition and calculation as it is faster to analyze four images at once. Zooming is advantageous provided that each object of interest is still

covered by a minimum number of pixels (> 100) to ensure the quality of the results (see section 3.1.1).

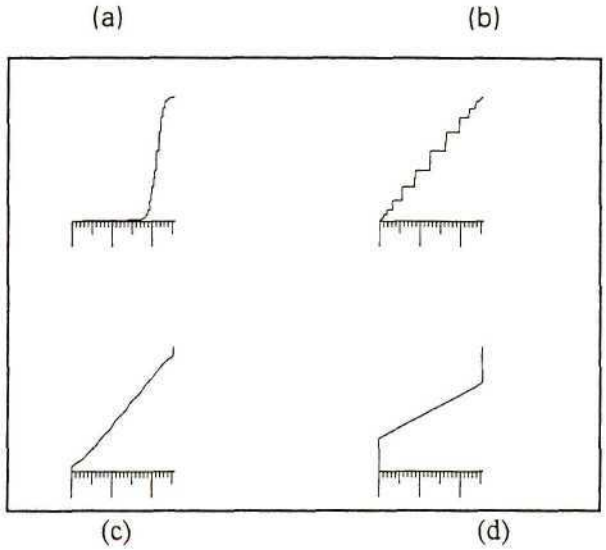


Figure 3.6. Grey-level frequencies of Figure 3.5 images from original to filter enhancement. a. Original image. b. Filter enhanced (normalized). c. Background corrected. d. Filter enhanced (filter "enhance contour").

x. Segmentation

The grey-level image held within Vidas was converted to a binary image (on which measurements could be made) by highlighting pixels above a given threshold intensity and converting the remainder to the background level (black). This was done in the macro "-seg_1" by two-level discrimination (selecting 2 levels, an upper and lower, from possible 0 - 256 grey levels), interactively or automatically by multi-phase segmentation, with the resultant production of a binary image (Figure 3.7) (Appendix Three, p128). Automatic discrimination was faster and preferred but had to be checked to ensure the desired objects were selected. Where the image was difficult to discriminate then interactive discrimination was used. In the macro "-seg_2" multiple images were segmented with the added benefit of checks (contour overlay, see section 3.3.6.i) and correction facilities (cutlink function).

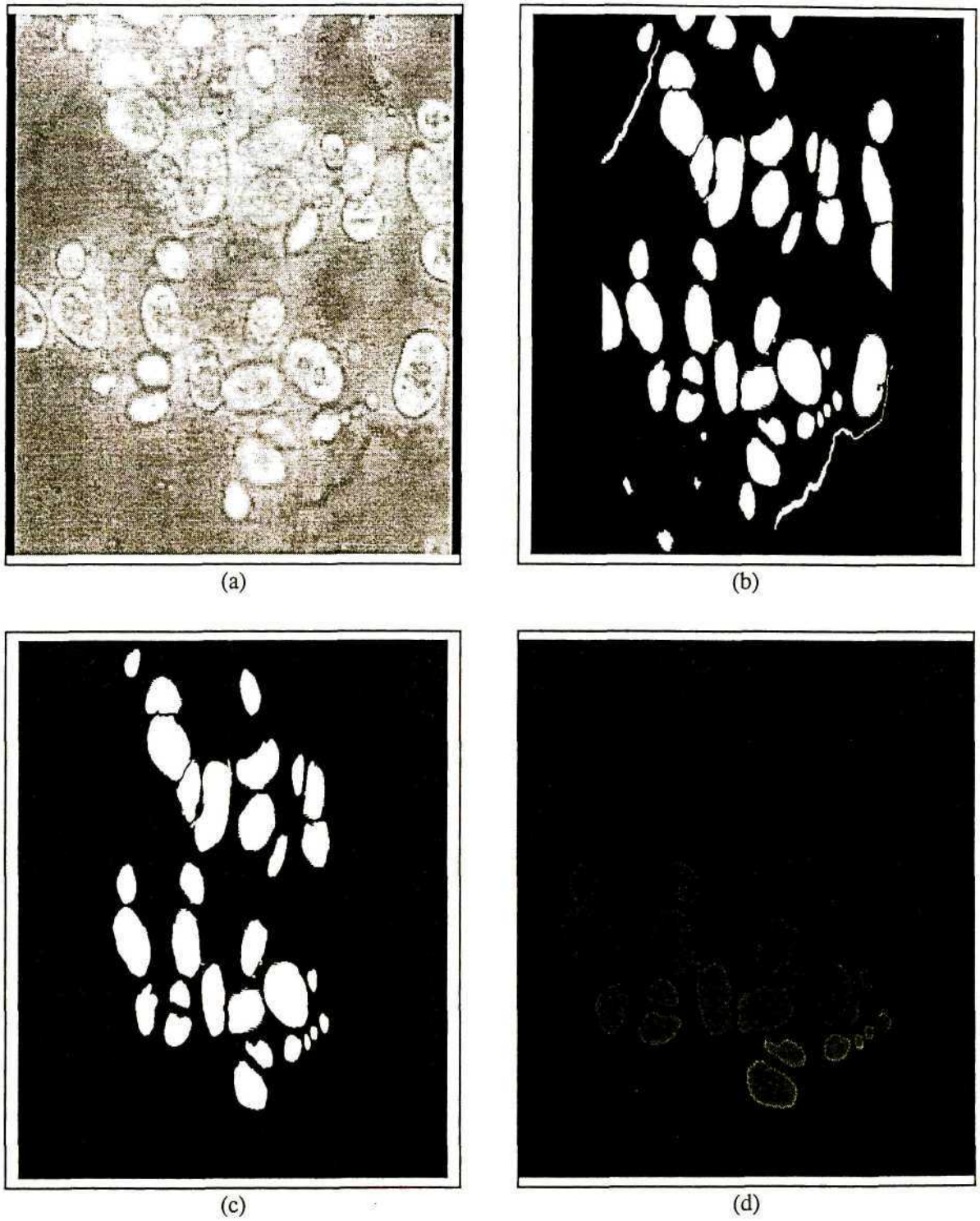


Figure 3.7. Typical image of cells, examined with a Kontron Vidas 2.1 Image Analyzer, showing segmentation, scrap function and frame correction. a. Original image. b. Segmented image. c. Scrapped image at 100 pixels. d. Identified image with frame correction.

xi. Binary Image Processing

The binary image may be edited with the cursor and digitizer tablet during an interactive step by comparison with the original video image. Editing would include the removal of remaining detrital particles, separation of adjoining bacteria, etc. The processing consists of one or a combination of the following: erosion, dilation, opening, closing, scrapping, marking, thinning, filling, link lines and reject or cut-linking objects of different shapes, phases, counts, areas or steps. The two most frequently used filters are scrap (eg. < 100 pixels on the screen) (Figure 3.7c) and cutlink. The scrap function allows objects smaller than a particular threshold size, eg. 20 pixels, to be removed from the screen while the cutlink function separates adjoining objects.

xii. Identification

A frame mode of identification was used with the macro "-indent". The size of the frame (objects outside the frame are removed) from the edges of the image was determined from the size of the microorganism (DMAX) (Appendix Three, p126). The frame mode was set to include microorganisms within the frame (Figure 3.7b/c). If this facility is not used, the results would be biased by parts of bacteria which were cut off when the image was captured. These edge-affected bacteria would result in a smaller determined size if included. This function may negatively affect large objects at a high magnification but this was resolved by performing the analysis at a lower magnification.

xiii. Measurement

Using an identified image and the macro "-measf" (Appendix Three, p126), field-specific measurements (based on the whole image) were made first, followed by object-specific measurements and finally, if required, densitometric measurements. Once measurements were completed, the results were filed into different data bases depending on the criteria chosen to separate the different microorganisms (Figure 3.8). In a typical example the criteria used to identify coccal type cells were an area greater than and an fcircle (circularity) less than anything else in the field. The rods were selected on elongation (length) and an area greater than cocci but smaller than fungal spores or hyphae. Fungal spores were selected at the greatest area and a high fcircle.

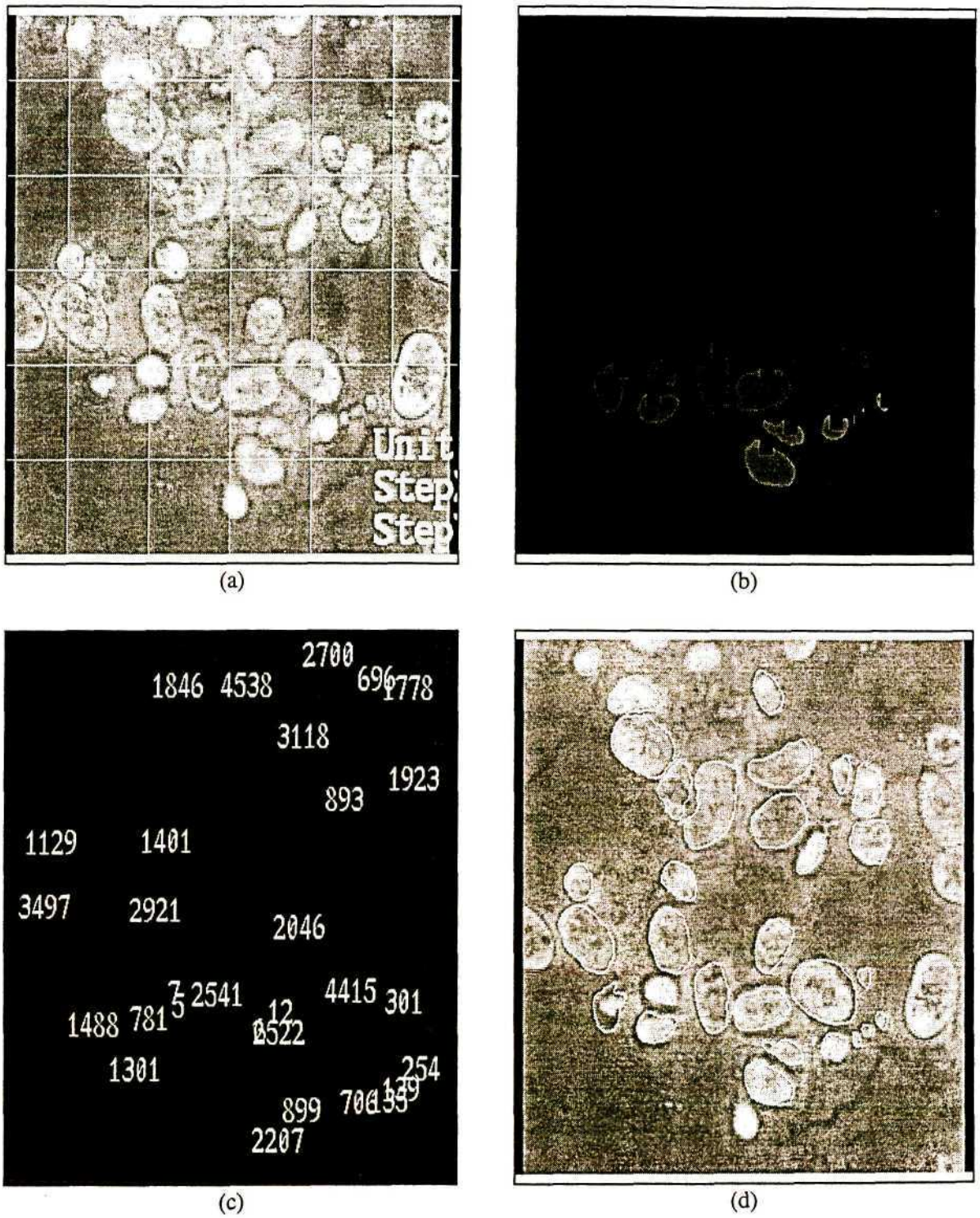


Figure 3.8. Typical image of cells examined with a Kontron Vidas 2.1 Image Analyzer. a. Original image overlayed with the scale. b. Object classification displayed by colour labels. c. Results displayed as area written to object. d. Overlay of (results of) the measured image over original image.

Fungal hyphae were selected by having the greatest elongation and an area greater than that of bacterial rods. The macro "-smdbp_1" allowed the setting up of the different data bases, measurement and storage based on selected criteria with the results displayed on the screen (Figure 3.8) (Appendix Three, p128).

xiv. Acquisition of Results

The object results (measurements which relate to the individual objects in an image) of each image were stored in the first set of data bases, with the results of the different types of microorganisms stored in different data bases (Appendix Three, p127). Following completion of image measurement the data's transferred to the second object data bases where all the results of that investigation are stored. A user-defined value (referring to the image number) is entered at this point. The first set of object data bases is then used for the results of the next image, to check the accuracy of the results they are written to the computer screen. The operator then checks that a coloured square is placed over each object measured. The colour indicates the different types of microorganism (and hence the data base to which it is written. An area, fcircle or elongation result is written to the screen in order to check and eliminate mistakes. The results may be listed, printed, a histogram constructed or the result data bases can be saved and exported to Videoplan (Kontron) for statistical analysis. From here they may be exported to Lotus, Data base or a free format for further analysis.

An important aspect of the results of image analysis is the production of good quality images to display the results obtained. A significant limitation of the Kontron IA system used in this study was in the production of digital printed images of limited quality. As the software only printed to 150 dpi the quality of the images obtained was low. Images of improved quality can be produced by the use of a normalization function during printing (in this case normalization provided greater contrast). The software limitation of 150 dpi was the significant element. With good software, very impressive images can be produced at 600 dpi.

3.3.6 **Description of Programmes**

Introduction: The programmes listed in Table 3.2 (Appendix Four, p130) were created specifically to analyse multiple images. These programmes are a combination of created macros and individual commands used to cycle through large numbers of images . These programmes are particularly useful for applications where user input can be limited or restricted to specific aspects. The programme "background correction" allows multiple image retrieval, background correction and storage of new images. Background correction of a single image may take > 45 minutes. It was, therefore, important to be able to run automated programmes overnight. The large number of images involved meant that even rapid steps, when multiplied by 200 times, would be time consuming. The macro "automatic processing" allows substantial automatic processing of images. Image enhancement was essential to permit recognition of many of the bacteria in the images. The macro "image enhancement" allows large-scale addition, retrieval and storage of images. Image segmentation was a manually intensive and slow process. The macro "segmentation" facilitated intensive segmentation of many images within a short time.

The most important step in the entire process of image analysis of biofilms, that of measurement, was covered by three macros. The macro "measurement macro 1" facilitates the automatic measurement of segmented images. "measurement macro 2" was used to selectively measure microorganisms in segmented images while "measurement macro 3" was used for the measurement of large numbers of segmented images. The macro "reproducibility" was used to check the accuracy of the image analyzer (see section 3.3.3).

i. Demonstration Macro One

As a means of showing how the images were analyzed three demonstration macros are described (Table 3.3 and Appendix Five, p133). Demonstration macro one was used for single images only. For single image quantification the IA started with an image either captured off video or from storage . This grey-level image (Figure 3.9a) was then retrieved from the Bernoulli disk, normalized or linearized to improve the contrast (Figure 3.9b), background corrected (Figure 3.9c) and the edges enhanced (Figure 3.9d). A binary image

was then created by segmentation (Figure 3.10a). This image was then scrapped (Figure 3.10b) before identification and measurement.

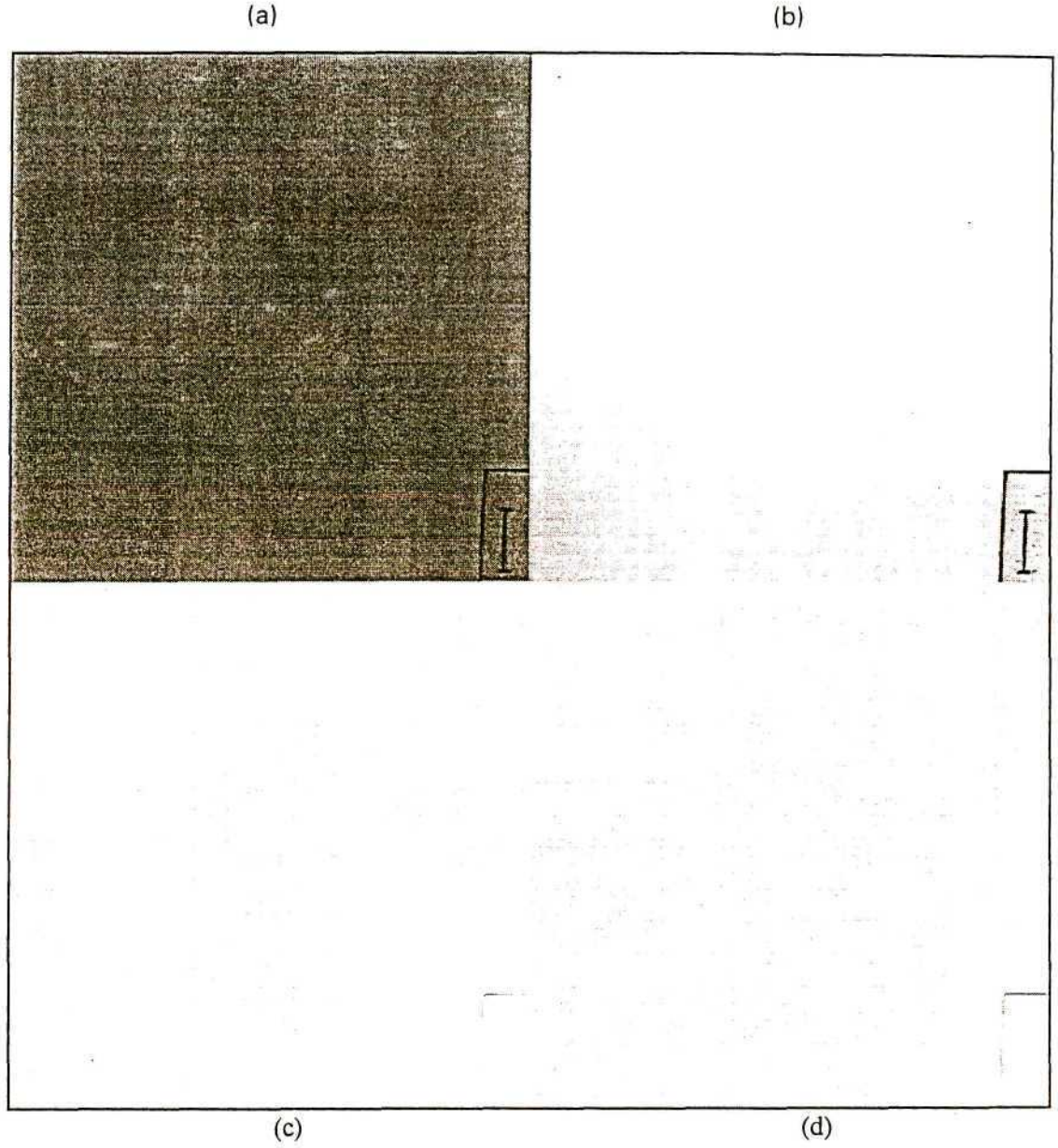


Figure 3.9. Images from demonstration macro one in which an image obtained with a Kontron Vidas 2.1 Image Analyzer was retrieved from a Bernoulli disk, linearized to improve the contrast, background corrected and the edges enhanced. a. Original image. b. Linearized image. c. Background corrected image. d. Edges enhanced.

The results were written onto the original image, which was overlaid with the image used for measurement as a validation (Figure 3.10c and 3.10d). This validation was essential to ensure that no microorganisms were missed or that objects which were not microorganisms were measured.

ii. Demonstration Macro Two

Demonstration macro one was useful for single images but proved tedious when large numbers of images were examined due to the excessive time required to process each image. Demonstration macro two solved this problem by allowing multiple images to be measured with minimal operator intervention. This effectively separated the time-intensive procedures from the user-intensive procedures, allowing optimum utilization of time on the image analyzer. The optimum utilization of time was an important aspect of the programmes due to: 1) the complex nature of the images; 2) the large number of images which had to be analyzed; 3) the limited computing power of the central computer; and 4) the work pressure on the image analysis apparatus. Demonstration macro two allowed the retrieval of numerically increased image numbers, partial setup, automatic scale setting, opening of data bases, measurement of objects and storage into different data bases, with classification shown on the image screen. The results were either printed, produced as histograms or exported to the required package.

iii. Demonstration Macro Three

Demonstration macro two was appropriate for multiple images where the scale was constant. Where one had to use different scales on the same or different images (because of different primary magnification), demonstration macro three was used. This allowed the retrieval of consecutively numbered images and the use of calibrated scales (with operator intervention). Depending on the scale various marker overlays were used as a visual check (Figure 3.11). The data base functions and results display were the same as macro two.

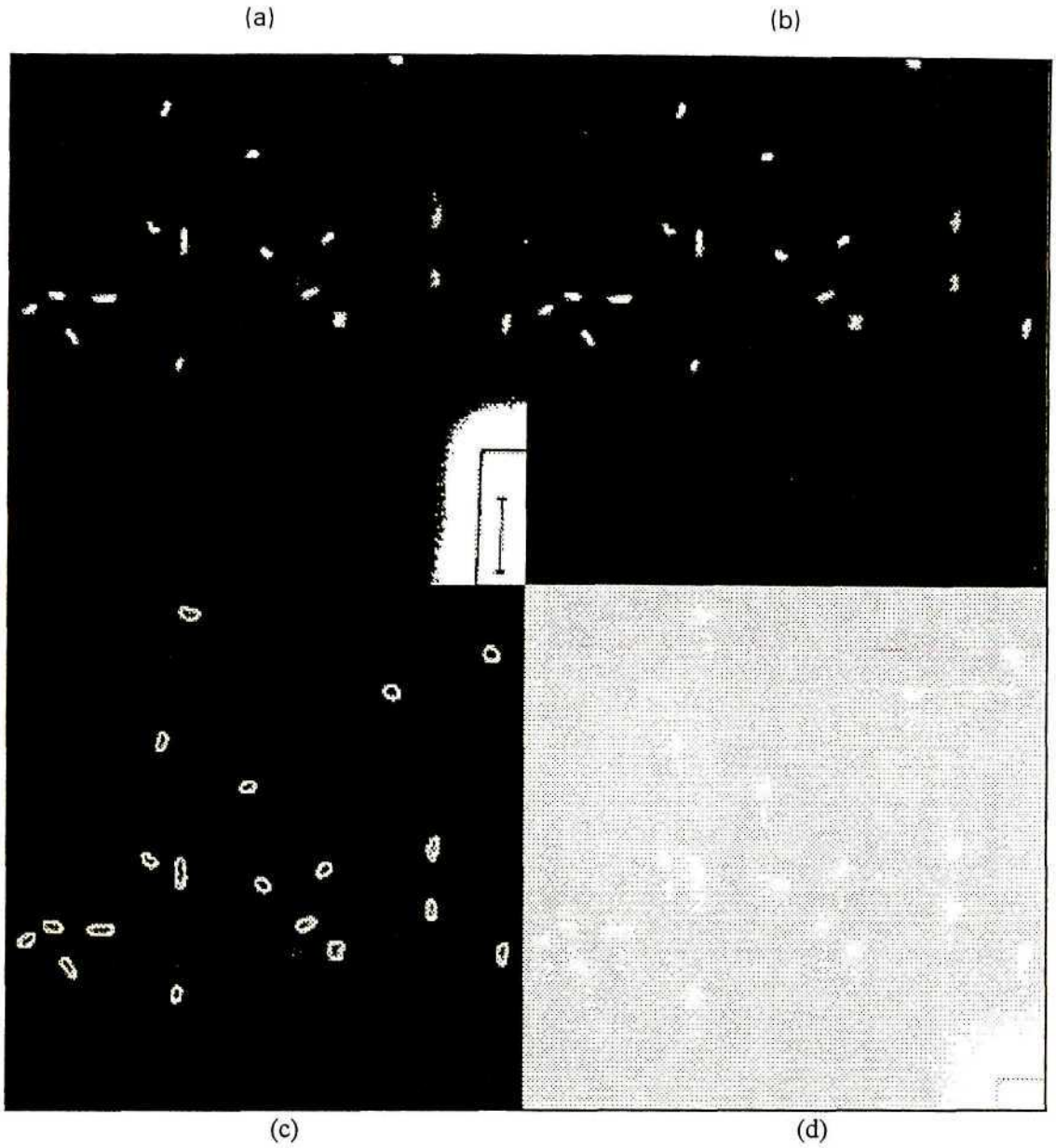


Figure 3.10. Images from demonstration macro one in which a binary image was created by segmentation, and scrapped before identification and measurement. The results were written onto the original image as a validation. a. Segmented image. b. Scrapped image. c. Overlay of measured image. d. Overlay of results (area) on original image.

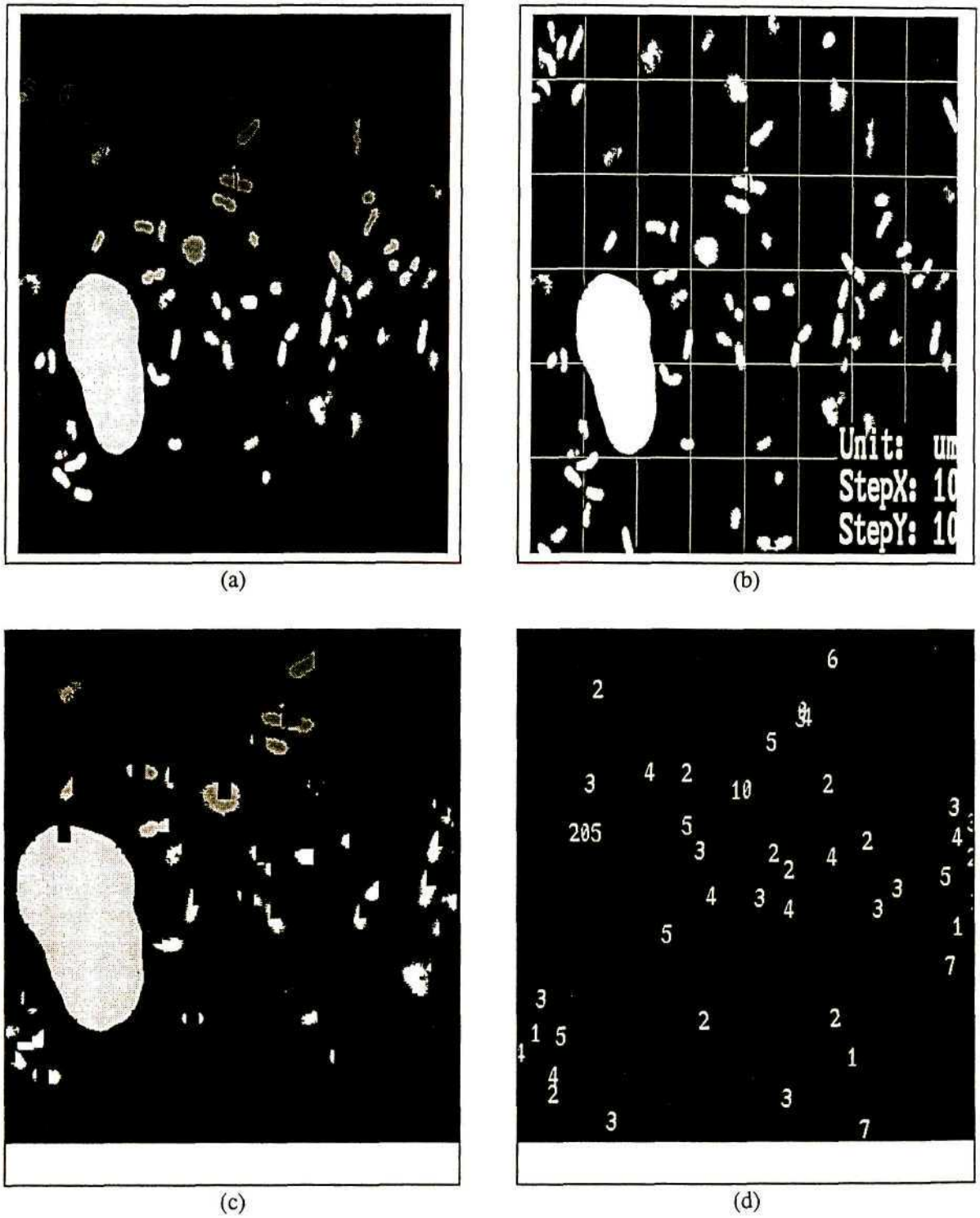


Figure 3.11. Images from demonstration macro 3, obtained with a Kontron Vidas 2.1 Image Analyzer. a. Original image. b. Original image overlayed with the scale. c. Object classification displayed by colour labels. d. Results displayed as area written to object.

3.4 *DISCUSSION*

The above applications show that image analysis facilitated progress in the fundamental research of landfill microbiology. The designed programmes were specific to the exploration of the interactions between isolated microorganisms, surfaces and the perturbant. More information may now be gained since the interactions can be visually observed in real time. There is no longer a rift between the experiment and the results since the microorganisms under study can be observed *in situ* and detailed measurements can be obtained.

The principal advantage of using image analysis in this project is in the quantification of images of undisturbed microbial associations. This represents a significant improvement on previous methods used to estimate *in situ* growth. The built-in image processing abilities allow image restoration and enhancement and thus permit the analysis of a large number of images.

There are, however, disadvantages when a visual technique is used. The possibility exists that the researcher is measuring an effect of the interactions taking place. Once one cannot use visual estimates real measurement ceases. While density measurements can help alleviate this problem, the solution may be to use a laser scanning microscope which can visualize below the surface of the biofilm.

Image analysis provides a technique to quantify the numbers and/or surface areas of bacteria in CCMU. The enumeration of filamentous fungi has generally been achieved through determining the length of hyphae rather than the number of individual cells (Packer and Thomas, 1990; Bolton *et al.*, 1991; Hitchcock *et al.*, 1996). Packer, Keshavarz-Moore, Lilly and Thomas (1992) in a study on bacteria and fungi examined both area and number and demonstrated that area was the parameter better suited to use for the isolated association, as used in this investigation. Thus, for any interacting microbial association it would be essential to check the merits of the different measurement parameters before use.

Image analysis can improve the quality of information available concerning microbial population dynamics within surface microenvironments (Murga *et al.*, 1995). In particular,

image analysis can increase the "effective" resolution of the light microscope beyond the 0.2μm range normally obtained under optimal conditions (Caldwell, Brannan, Morris and Betlach, 1981). This is done in two ways, firstly by image processing (primarily background correction and filters) which corrects the image appearance, and secondly, by the standardization of the edges of bacteria in low resolution images. While the latter may not be the true edge (i.e. not the actual size) it allows comparisons to be made (Bloem *et al.*, 1995).

In biofilm colonization studies, it is beneficial to retrieve images of the objects outside the depth of focus of the microscope objective. Lawrence *et al.* (1989) described a method of combining images to increase "effective" depth of field at high magnification. Many smaller cells were detectable in the resultant high contrast combined images. As a consequence, additional information on the extent of, for example, microbial colonization of soil surfaces, was possible (Wynn-Williams, 1990). In studies of microbial colonization, many replicate fields are quantified to ensure statistical significance. The task of down-loading the images into the image analyzer and the time taken to analyze each image, make this process very time consuming. To escalate this by a factor of three to ensure 3-D retrieval of all objects would be logistically difficult with the equipment used in this study. The combination of images provides a valuable correction factor for routine use of a single optimal focal plane when applied selectively to random representative replicate samples (Wynn-Williams, 1990).

Versatile image analysis macros were successfully developed to quantify initial microbial growth within CCMU's and on opaque surfaces. The methods developed were used to examine an hexanoate-catabolizing microbial association to define its stages of attachment and initial development under constant, standardized conditions (5.3). Although morphology alone is insufficient for precise identification (Westlake *et al.*, 1995; Posch *et al.*, 1997), the experiments provided some information on the possible types of bacteria present in an aerobic association isolated from refuse. The optimum dilution rate and substrate concentration were determined together with the specific growth rates of both the association and the component types (6.3).

CHAPTER FOUR

4. ENRICHMENT, ISOLATION, ENUMERATION AND CULTURE OF HEXANOIC ACID CATABOLIZING MICROBIAL ASSOCIATIONS

4.1 *INTRODUCTION*

4.1.1 Enrichment

Enrichment often involves the transfer of soil, sewage or some other suitable source of inoculum with a large and diverse population of microorganisms into a selective medium followed by incubation under conditions which favour growth of the desired species. In general terms, the greater the heterogeneity of the environment, the more diverse and complex the microflora (Slater and Hardman, 1982). For details of methods for enriching, isolating and analyzing microbial communities in laboratory systems see Parkes (1982).

4.1.2 Enumerating Microorganisms

Even in a laboratory, enumerating microorganisms and estimating microbial biomass are complex problems (White, 1983). The diversity of the microorganisms and the habitats in which they occur require the development and use of a variety of enumeration procedures (Fletcher, 1990; Herbert, 1990). There are no universal methods which can be applied to all microorganisms and all habitats. The enumeration procedure begins at the time of sample collection (Fletcher, Latham, Lynch and Rutter, 1980). Sampling and handling procedures must ensure that the number subsequently determined accurately reflects the number actually present in the habitat (Harvey and Young, 1980; Albrechtsen, 1994).

Viable plate count procedures are most frequently used for the enumeration of bacteria but may also be used for other groups of microorganisms, including algae and fungi (Herbert, 1990). The use of viable plate counting procedures for estimating fungi is, however, strongly biased towards an underestimation of the number (Postgate, 1969).

4.1.3 **Fluorescence Microscopy**

The use of fluorescence microscopy and fluorescent dyes, such as acridine orange, permit direct, accurate counting of even small bacteria (Trolldenier, 1973; Daley and Hobbie, 1975; Jiang, Wang and Li, 1995). Image analysis is an extension of this method (Farr, 1986). For increased contrast, dark (black-stained) filters are used (Jones and Simon, 1975; Zimmermann and Meyer-Reil, 1976). A significant factor in the successful use of direct epifluorescence is that, provided care is taken (Easter, Kroll, Farr and Hunter, 1987), good relationships between fluorescence and total aerobic plate counts can be obtained (Back and Kroll, 1991). Direct epifluorescence has often been preferred to scanning electron microscopy due to its specificity, ease of sample preparation (Van Es and Meyer-Reil, 1982) and better size determinations (Fuhrman, 1981). Direct epifluorescent counts can be accepted as good estimates of total number but cannot be used to determine if the microorganisms are living or dormant.

4.1.4 **Continuous Culture**

The complexity of environments such as a municipal solid waste landfill, in which bacteria thrive, has prompted microbial ecologists to develop experimental systems to study the behaviour of microorganisms under well-defined and controlled conditions (Brown and Williams, 1985). Often bacteria in continuous culture are used to ensure metabolic uniformity (Patrick and Larkin, 1993) and to avoid the intrinsic sampling, monitoring and other problems which occur with *in situ* investigations (Slater and Hardman, 1982; Brown, Costerton and Gilbert, 1991). The basic continuous culture system is characterized by a culture vessel, the content of which is well mixed, to which fresh medium is added at a constant rate while the working volume is kept constant via an overflow device (Evans, Herbert and Tempest, 1970). The bioreactor is, thus, an open system. All the components required for growth are present in excess in the medium, except one, the growth-limiting substrate (Herbert, Elsworth and Telling, 1956). Ideally, this cultivation system allows continuous exponential growth of microorganisms under constant environmental conditions (Gottschal and Dijkhuizen, 1992). It is designed to create a self-adjusting steady-state condition which is ideally suited for physiological studies since it provides a most convenient way of keeping microorganisms in a well-defined physiological condition over long periods of time (Jannasch, 1965; Gottschal, 1990). Bacterial surface

structures may be subject to environmentally induced variation *in situ* and surface structures may be different from those in laboratory culture (Slater and Hardman, 1982; Sutherland, 1977).

This single-stage, flow-controlled system is the cultivation technique best suited for the study of a wide spectrum of microbiological problems (Dykhuizen and Hartl, 1983). For extensive discussion of continuous culture refer to Tempest (1970) and Gottschal and Dijkhuizen (1992) and for detailed practical information see Evans *et al.* (1970) and Gottschal (1990). In principle, a microorganism multiplying in a continuous culture grows at a constant sub-maximal specific growth rate (μ) when the rate of dilution (D) of the culture with fresh medium is constant. Under these conditions a steady state is established where μ = D (Gottschal and Dijkhuizen, 1992).

4.1.5 **Objectives**

The objective of this phase of the research programme was to enrich, isolate, enumerate and maintain in aerobic or anaerobic continuous culture microbial associations from landfilled refuse.

4.2 *MATERIALS AND METHODS*

4.2.1 **Inocula: Sampling, Storage and Preparation**

To enrich and isolate hexanoate-catabolizing associations three sources of inoculum from the Umlazi Landfill, South Africa, were used: One. Soil cover (Dudley *et al.*, 1994a); Two. Soil from the landfill base liner where gas and leachate were actively emitted; and Three. Municipal refuse (Glover, 1992).

Two samples (1kg each) of soil from each source were collected with a hand auger. The samples were placed in plastic bags which were then sealed and transported to the laboratory where they were stored at 4°C for periods not exceeding 3 days.

Municipal refuse was sampled from a depth of 2m from an area that had been landfilled six months previously. Two samples (10kg each) were placed in plastic bags which were

then sealed and transported to the laboratory where they were stored at 4°C for periods not exceeding 3 days.

4.2.2 **Mineral Salts Solution**

The mineral salts solution used was the same as that described by Watson-Craik (1987) and contained (gl $^{-1}$ distilled water): K_2HPO_4, 1.5; $MgCl_2.6H_2O$, 0.2; $NaH_2PO_4.2H_2O$, 0.85; Na_2SO_4, 1.4; NH_4Cl, 0.9; $NaHCO_3$, 0.5; Na_2CO_3, 0.3, $NiCl_2.6H_2O$, one mM, one ml; vitamins, one ml; trace element solution A, one ml; trace element solution B, one ml.

Vitamins: The vitamins used in the mineral salts were (mgl $^{-1}$ distilled water): biotin, 10; *p*-aminobenzoic acid, 10; folic acid, 10; pyridoxine HCl, 20; thiamine, 20; riboflavin, 30; nicotinic acid, 50.

Trace Element Solution A: Trace element solution A contained (mgl $^{-1}$ distilled water): $FeCl_2.H_2O$, 1500; NaCl, 9000; $MnCl_2.4H_2O$, 197; $CaCl_2$, 900; $CoCl_2.6H_2O$, 238; $CuCl_2.H_2O$, 17; $ZnSO_4$, 287; $AlCl_3$, 50; H_3BO_3, 62; $NiCl_2.6H_2O$, 24; conc. HCl, 10ml.

Trace Element Solution B: Trace element solution B contained (mgl $^{-1}$ distilled water): $Na_2MoO_4.2H_2O$, 48.4; $Na_2SeO_3.xH_2O$ (31%Se), 2.55; $Na_2WO_4.2H_2O$, 3.3.

4.2.3 **Mineral Salts Solution Preparation**

Volumes of mineral salts were prepared in three stages. A $NaHCO_3$, Na_2CO_3, trace element and vitamin deficient solution (900 ml) was diluted to one litre with distilled water. This was dispensed into flasks which were then closed with non-absorbent cotton wool bungs and aluminium foil, and autoclaved (BUTTERWORTH, Butterworth Productions (PTY) Ltd, South Africa) at 121°C (15lb psi) for 15 minutes. Appropriate weights of $NaHCO_3$ and Na_2CO_3 were added to 97 ml of distilled water and the solution was sterilized by autoclaving as above. Finally, prior to use of the solution, 1ml each of trace element solutions A and B and vitamins, together with appropriate amounts of electron acceptors and the carbon source, were filter sterilized, by passage through a 0.2μm Millipore membrane filter, and added to the medium. The anaerobic solution was also supplemented

with resazurin (0.0005%, $w.v^{-1}$) (Postgate, 1966). Dispensing occurred under aseptic conditions on a laminar flow bench (SLEE, London).

4.2.4 Substrate

Hexanoic acid (caproic acid) was chosen as the oxidizable carbon source. Hexanoic acid has the following characteristics: $C_6H_{12}O_2$; C = 62.04%, H = 10.41% and O = 27.55%; oily liquid with molecular weight = 166.16. It is slightly soluble in water with a solubility of 10.82gl^{-1} (at 25°C) (Merck Index, 1996). It was added last to the mineral salts solution before the experiments.

4.2.5 Aerobic and Anaerobic Enrichments

Both aerobic and anaerobic enrichments were initiated at a pH of 7.2, with 5, 25 and 50mM final concentrations of hexanoic acid, at 30°C (Fielding, Archer, de Macario and Macario, 1988) in a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc, Edison, N.J.). The concentrations of hexanoic acid used were chosen from the concentration range found in landfill leachate (Harmsen, 1983).

A 1:10 ratio (g soil:ml medium) was used in the aerobic enrichments while a 1:3 ratio was used in the anaerobic enrichments. The anaerobic cultures were made in medical flats (230ml), filled to a 1:10 ratio of soil/liquid to gas, and overgassed with oxygen-free nitrogen (OFN) (FEDGAS) for 15 minutes. The medical flats were closed with Suba-seals which had been made air tight with silicone on both the internal and external surfaces. Hypodermic needles attached to 10ml syringes were used to collect and release accumulated gas produced as a result of microbial catabolizm.

The aerobic cultures were made in 100ml conical flasks which were shaken at 150rpm in an incubator shaker (New Brunswick Scientific Co. Inc, Edison, N.J.). Samples (5ml) were taken for analysis every 10d with a sterilized syringe. Once 90% of the original hexanoic acid had been utilized the cultures were resupplemented to the original concentration. This was done twice prior to subculturing. Subculturing consisted of inoculating 10% of the enrichment culture into fresh medium. Enrichments for each acid concentration and each

culture type (aerobic/anaerobic) were made in duplicate. For each duplicate the culture which effected the highest substrate catabolizm was used in the subsequent stage.

After the first subculturing, only the 25 and 50mM enrichments were continued. After the second subculturing the aerobic 25mM cultures were supplemented to hexanoic acid concentrations of either 50mM or 25mM. The aerobic cultures were subcultured twice while the anaerobic cultures were subcultured once.

At each subculturing stage MacCartney bottles which contained media supplemented with the appropriate concentration of hexanoic acid were inoculated (10%v.v^{-1}), allowed to grow and then stored in a refrigerator (4°C).

4.2.6 **Continuous Culture Units (CCUs)**

Once hexanoic acid-degrading associations (50mM) had been isolated they were inoculated (10%v.v^{-1}) into continuous culture units (CCUs) (D = 0.1h^{-1}) for long-term maintenance under anaerobic or aerobic conditions (Herbert *et al.*, 1956). The glass CCUs were 215mm long, with an internal diameter of 50mm (Plate 4.1).

Operating Conditions: The operating volume of each CCU was 135ml. The CCUs were maintained at 30°C in a temperature-controlled box (light excluded) (Plate 4.1). The aerobic cultures were aerated and mixed by bubbling filtered air (1 v.v^{-1}.min^{-1} aeration) through a drawn-down 1mm glass tube (Brown and Gilbert, 1993) while the anaerobic cultures were overgassed with oxygen-free nitrogen. The influent CCU hexanoic acid concentration was determined when the reservoir was changed.

Sampling: Samples (5ml) were taken via septa in the side ports of the units.

Influent Medium: Fresh medium supplemented with 50mM hexanoic acid (aerobic) or 10mM hexanoic acid (anaerobic) was added with a peristaltic pump (Gilson Minipuls 3 MP4) continuously to the top of each CCU.

Monitoring the Continuous Culture Units: Once the associations had been inoculated into the CCUs the influent and effluent of each unit were monitored. Residual substrate was determined by GC analysis (see section 2.1.1), microbial population sizes by turbidity readings (see section 2.1.2), number of microorganisms by image analysis (see section 3.3) and number of viable microorganisms by plate counts (see section 2.1.2).

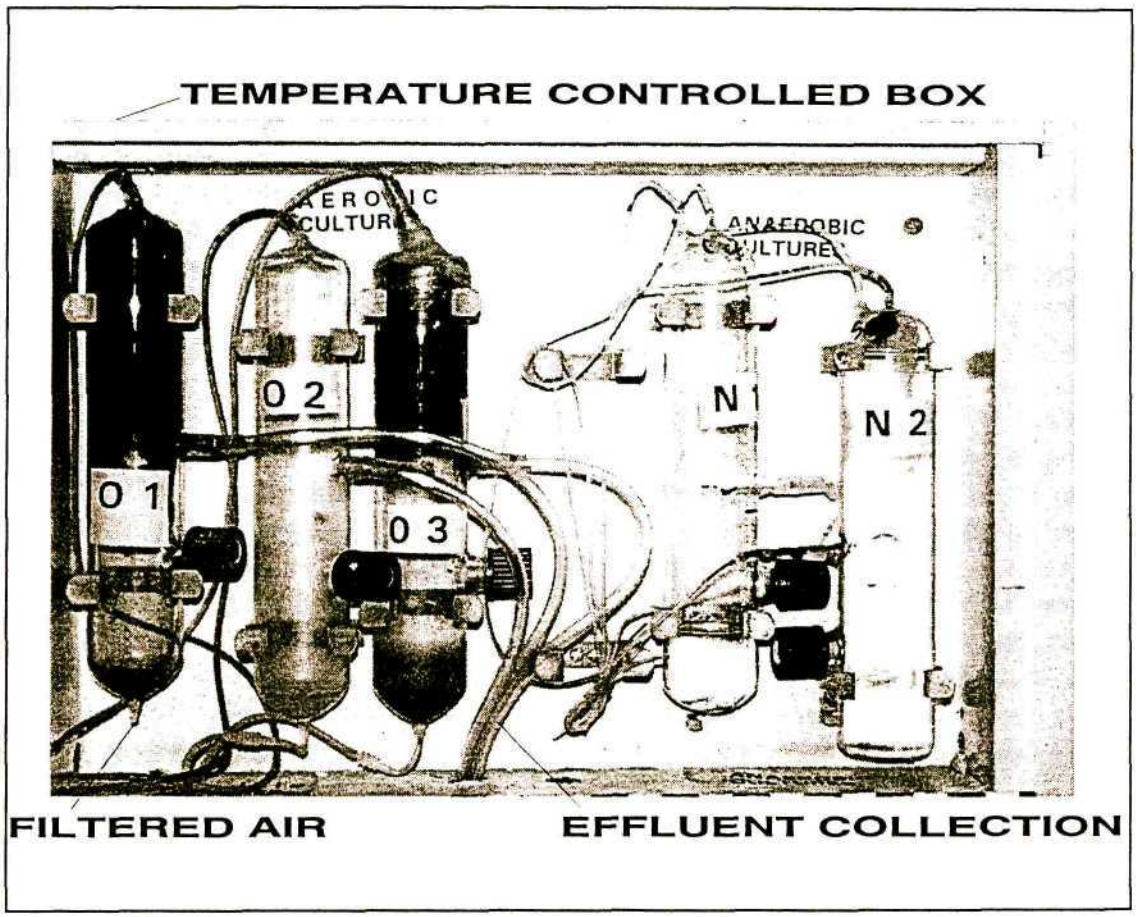


Plate 4.1. **Continuous culture units maintained in a temperature-controlled box used to maintain aerobic and anaerobic cultures of microbial associations enriched and isolated from municipal refuse (03), landfill cover (01) and base liner soils (02). The aerobic cultures were aerated by bubbling filtered air (1 $v.v^{-1}.min^{-1}$ aeration) through the medium supplemented with 50mM hexanoic acid. The duplicate anaerobic cultures (N1 and N2) were over-gassed with oxygen-free nitrogen (OFN) to maintain anaerobic conditions.**

4.2.7 Continuous Culture Microscopy Units (CCMUs)

The Continuous Culture Microscopy Unit (CCMU) (Figure 4.1) design was based on Berg and Block (1984) and Jones, Watson-Craik and Senior (1992a). Each CCMU was built by the Mechanical Instrument Workshop (Faculty of Science, University of Natal, Pietermarizburg) and consisted of a microscope slide onto which a spacer was glued followed by a long cover slip to form a chamber with internal dimensions of: length, 34mm; width, 5mm; and depth, 0.5mm. The total volume was 0.085ml.

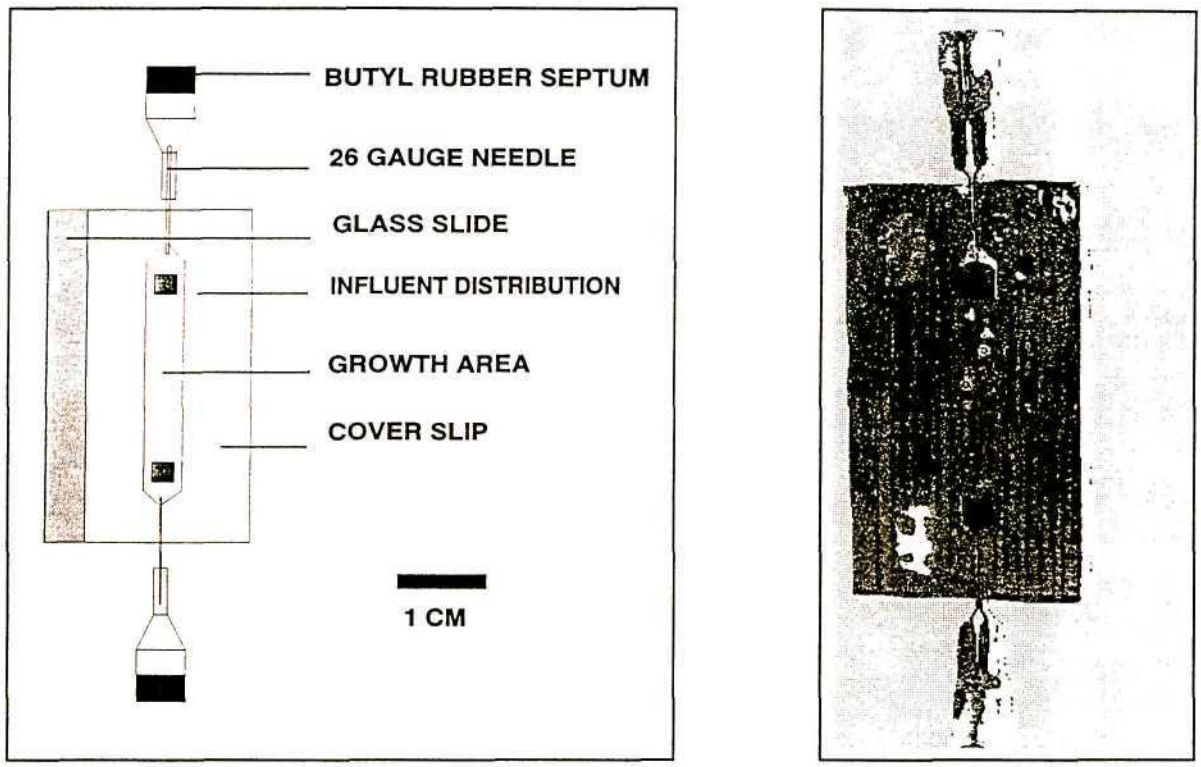


Figure 4.1. **Continuous Culture Microscopy Unit (CCMU) used to cultivate microbial associations for enumeration by image analysis. 4.1a Schematic representation. 4.1b CCMU and microscope holder. The microbial associations were isolated from a municipal solid waste landfill.**

Preparation of Surfaces: The internal CCMU surfaces were cleaned with acid-alcohol (3ml conc. HCl in 100ml 95%v.v^{-1} ethanol) prior to assembly. The internal surfaces were then oxidized and sterilized by baking in an oven (Gallenkamp) for 12h at 205°C before purging with the medium for five minutes to facilitate adsorption of solutes to the chamber surfaces prior to inoculation (Dudley ,du Plessis and Senior, 1994b). Influent/effluent were introduced/removed by a peristaltic pump (Ismatec IPN 24B) via silicon tubing (1 mm i.d.) attached to hypodermic needles (26 gauge) which were sealed with butyl rubber septa which tightly fitted into the plastic base of the needles (Figure 4.1). This allowed the CCMU to be removed from the temperature controlled box with ease without introducing air. To enable return (X,Y position) to a specific microbial cell in the CCMU, each CCMU was placed on a holder which fitted on the stage of the microscope.

The flow direction was from Image one (influent port) to Image 110 (effluent port) which was the point of inoculation. The CCMUs were maintained in a temperature controlled box (Plate 4.2) (Mechanical Instrument Workshop, University of Natal, Pietermarizburg) at 30°C and light was excluded (Dudley, 1993). Inoculum (microbial associations, 100% or 10%v.v^{-1}) was introduced into the outflow end with a sterile hypodermic needle and syringe (1ml). The CCMUs were left for 12 or 24h before applying the influent flow.

Sampling and Data Analysis: Individual sampling (due to the number of samples within a close proximity) was carried out unless indicated. The following terms relating to data analysis are defined and units indicated:

Area	-	Number of pixels multiplied by scaled pixel area (micrometers).
Circularity (fcircle)	-	Perimeter squared divided by four times area (ration with 1 being a straight line).
Discriminated phase.field^{-1}	-	All discriminated objects will be measured as none can be measured without discrimination, (pixels but often converted by scale to micrometers).
Elongation (length)	-	Diameter maximum divided by minimum diameter, (ratio).
Colonized area (average).	-	Area colonised as individual cells if four distinguished microbial cells could be determined or as an area occupied by the attached population of cells of each microbial morphotype or as a microbial consortium (micrometers).
Numbers of microroganisms.field^{-1}	-	Morphologically identifiable association members are counted and averaged per field examined.
Optical density.field^{-1}	-	densities (grey-level value) of the cells and or area of interest (other density measurements: GREYM = Mean grey value inside object; GREYSD - Standard deviation of grey value; TRANSMM = Mean transmission of object (%); TRANSMSD =Standard deviation of the transmission; OPTDM = Mean optical density; OPTDSD = Standard deviation of the optical density; OPTDI = Integrated optical density (OPTDI = OPTDM X AREA).
Total (SUM) area	-	Sum of the components which is calculated from all objects in the field of view (total field); includes items (such as EPS) which may not be morphologically identifiable association members (micrometers). An example would be the area of fungi (a more accurate measurement) compared to individual lengths of hyphea (unless a specific fungus orientated programme is used).

Biofilm Monitoring: At recorded times the CCMUs were removed from the holders in the temperature-controlled box and placed in the microscope holders. Maximum magnification, 1000X (7300X on IA computer screen with a Zeiss Axiophot microscope) (see section 2.1.3), equivalent to 45.4834μm^2, was used to examine bacterial rods and cocci and fungal spores and hyphae. A magnification of 400X (2920X on the screen with a Zeiss Axiophot microscope), equivalent to 4920μm^2, was used for microbial colonization studies. For assessing biofilm development a constant field at a specific Y axis point was captured on video tape (see section 3.2.4.iii). The video recorder continued recording while the next image was located. A minimum of 15s was used to capture each image. On average, 110 images were captured from each CCMU. When the biofilm became too thick for through-viewing, destructive analysis, by staining with acridine orange and viewing with fluorescence microscopy (see section 2.1.3), was made.

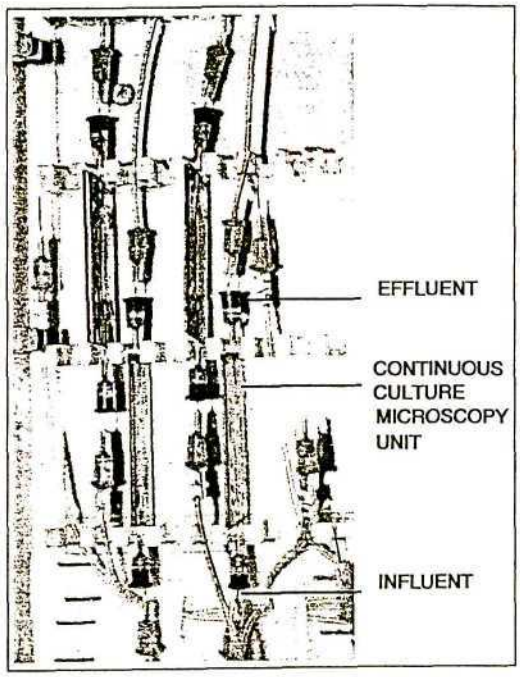


Plate 4.2. Temperature controlled box for housing CCMUs. Fifteen CCMUs (three rows, five CCMUs per row) were maintained at a constant temperature (30°C) without light.

4.3 *EXPERIMENTAL RESULTS*

4.3.1 Enrichment and Isolation

Hexanoic acid is a free volatile fatty acid (see section 4.2.4) and was chosen as the oxidizable carbon source as it is a representative low molecular weight molecule and is a constituent of landfill leachate, especially in the acidification stage (Harmsen, 1983). In

an enrichment a discrete concentration of hexanoic acid is the summation of the concentration present in the inoculum source material, the concentration generated from the inoculum source material, the concentration added and the concentration catabolized by the inoculum. The results quoted are averages of duplicate analyses. The soil cover (see section 4.2.1) was Inanda top soil which was to be used as daily, intermediate and final cover of the refuse.

With 5mM hexanoic acid supplementation and refuse inoculum the initial concentrations of hexanoic acid detected were much greater (over 2.5 times) than the concentration added. Inoculum three contained, on average, 0.295g hexanoic acid.g^{-1} refuse which equated to a culture supernatant concentration of 9.3mM. The soil from the landfill base liner where gas and leachate were actively emitted contained, on average, 0.026g hexanoic acid.g^{-1} soil which equated to a culture supernatant concentration of 4.1mM hexanoic acid before supplementation. It is possible that these supernatant concentrations resulted through desorption when mineral salts were added. The hexanoic acid may, therefore, have been made available to free-living microorganisms.

i. Aerobic Cultures

With the aerobic batch cultures, Inoculum three (municipal refuse), in the presence of the highest concentration of hexanoic acid (50mM), effected the shortest lag phase for hexanoic acid catabolizm. The hexanoic acid was degraded in 10 - 20d. This was to be expected since refuse either contains or generates hexanoic acid as a catabolic intermediate thus providing the selection pressure for the enrichment of hexanoate catabolizing microbial associations.

The two soil inocula, in the presence of five and 25mM hexanoic acid supplementation, took 20d to effect close to 90% degradation. This indicated that soil (Inoculum one) without previous exposure to high concentrations of hexanoic acid contained associations which had the ability to degrade the molecule. Faced with the same selection pressures *in situ*, similar associations should then be able to reduce the inimical threat of the acid to plant species provided that the hexanoic acid concentration did not exceed a concentration which is inhibitory (K_i) to the catabolic species. Within 10d of subculturing the aerobic enrichments (see section 4.2.5) in the presence of 25mM hexanoic acid > 90%

catabolizm had been effected in each of the cultures. The 50mM supplementations were considerably slower, with < 80% degraded after 20d.

For the second subculturing of the 25mM hexanoate cultures the hexanoic acid concentration was increased to 50mM. The 25mM hexanoic acid supplementations were, however, continued for the eventuality that the 50mM supplementations proved inhibitory. The results of increasing the hexanoic acid concentration to 50mM showed that, even with twice the initial concentration, only limited residual substrate remained (< 9%) after 20 days. The original 50mM enrichment cultures also continued to degrade the hexanoic acid but at a much slower rate (after 20d < 70% was degraded) than the original 25mM supplemented cultures. This suggested that in the refuse mass/landfill bioreactor exposure to low concentrations (≤ 25mM) of hexanoic acid over a short time period should facilitate enrichment of an active catabolic association, provided that oxygen is non-limiting. Exposure to higher concentrations (> 25mM) of hexanoic acid may, however, result in protracted lag phases.

ii. Anaerobic Cultures

The trend of the 25mM hexanoic acid-supplemented culture effecting the shortest lag phase continued with the anaerobic batch culture enrichments. Inoculum two (soil from the landfill liner where gas and leachate were actively emitted) and Inoculum three (municipal refuse) with five and 25mM hexanoic acid supplementations, respectively degraded the substrate comparatively rapidly (< 5% substrate remained after 30d). In the anaerobic batch cultures all the inocula degraded the five and 25mM hexanoic acid supplements. Only Inoculum one effected substrate catabolizm within 30d when the concentration was 50mM. With this concentration only limited catabolizm was recorded with Inocula two and three (< 20% and 30%, respectively) in the 30d incubation period.

Table 4.1 records the gas emission results of the enrichments. Possible substrate inhibition in some anaerobic cultures contrasted active gas evolution in others. In municipal landfills the refuse is mostly in an anaerobic state due to oxygen displacement and/or microbial utilization. Thus, if high hexanoic acid concentrations are present then subsequent catabolizm may be inhibited with resultant accumulation. Williams (1993) reported high background concentrations of hexanoic acid in similar refuse. If plant roots

from covering vegetation penetrate the anaerobic zone the hexanoic acid will negatively impact on growth (Williams, 1993). Inoculum one, however, in the presence of 50mM hexanoic acid supplementation catabolized the substrate (> 99% in 30d).

Table 4.1. Gas emissions, recorded by syringe plunger displacement and expressed as percentages of duplicates, from anaerobic batch culture enrichments supplemented with five, 25 or 50mM hexanoic acid and inoculated with: One. Soil cover; two. Soil from the landfill liner where gas and leachate were actively emitted; or three. Municipal refuse.

HEXANOIC ACID INITIAL ADDED CONCENTRATION (mM)	INOCULUM	GAS EVOLVED		
		10d	20d	30d
5	ONE	0%	50%	100%
	TWO	0%	100%	50%
	THREE	0%	100%	100%
25	ONE	0%	100%	100%
	TWO	50%	100%	0%
	THREE	100%	50%	100%
50	ONE	100%	100%	100%
	TWO	50%	0%	0%
	THREE	0%	0%	50%

When the anaerobic cultures were subcultured, catabolizm of 25mM hexanoic acid was rapid (> 97% in 10d) while the 50mM cultures took longer (< 56% in 20d). The gas emission results appeared to support the mineralization of hexanoic acid with more gas in general produced by the 25mM-supplemented cultures (Table 4.2).

Table 4.2. **Gas emissions, recorded by syringe plunger displacement and expressed as percentages of duplicates, from anaerobic batch subcultures (25 and 50mM hexanoic acid) of primary enrichments inoculated with: One. Soil cover; Two. Soil from the landfill base liner where gas and leachate were actively emitted; and Three. Municipal refuse.**

HEXANOIC ACID ADDED INITIAL CONCENTRATION (mM)	PRIMARY ENRICHMENT SOURCE MATERIAL	GAS EVOLVED		
		10d	20d	30d
25	ONE	100%	100%	100%
	TWO	0%	100%	100%
	THREE	100%	100%	50%
50	ONE	0%	0%	50%
	TWO	50%	0%	100%
	THREE	50%	100%	100%

Although the lag periods varied, catabolic associations for hexanoic acid resulted in every enrichment. Evans and Ahlert (1987) used industrial landfill leachate to quantify the degree of acclimatization of a mixed culture. They found that the acclimatization was dependent on the quality (diversity) of the inoculum although a poor inoculum did not preclude an acclimatization which resulted ultimately in a superior culture.

iii. Subculturing

After the first subculturing stage the anaerobic microbial associations were inoculated into the CCUs.

4.3.2 **Continuous Culture**

i. Influent and Effluent Substrate Concentrations

The influent hexanoic acid concentration was set at 50mM but varied from 45 mM to 55mM, with an average of 48.9mM, during the course of the study. The effluent concentrations of the aerobic CCUs are shown in Figure 4.2 from which it can be seen that Association three catabolized the substrate to a much lower concentration up to day 80 than the other two associations.

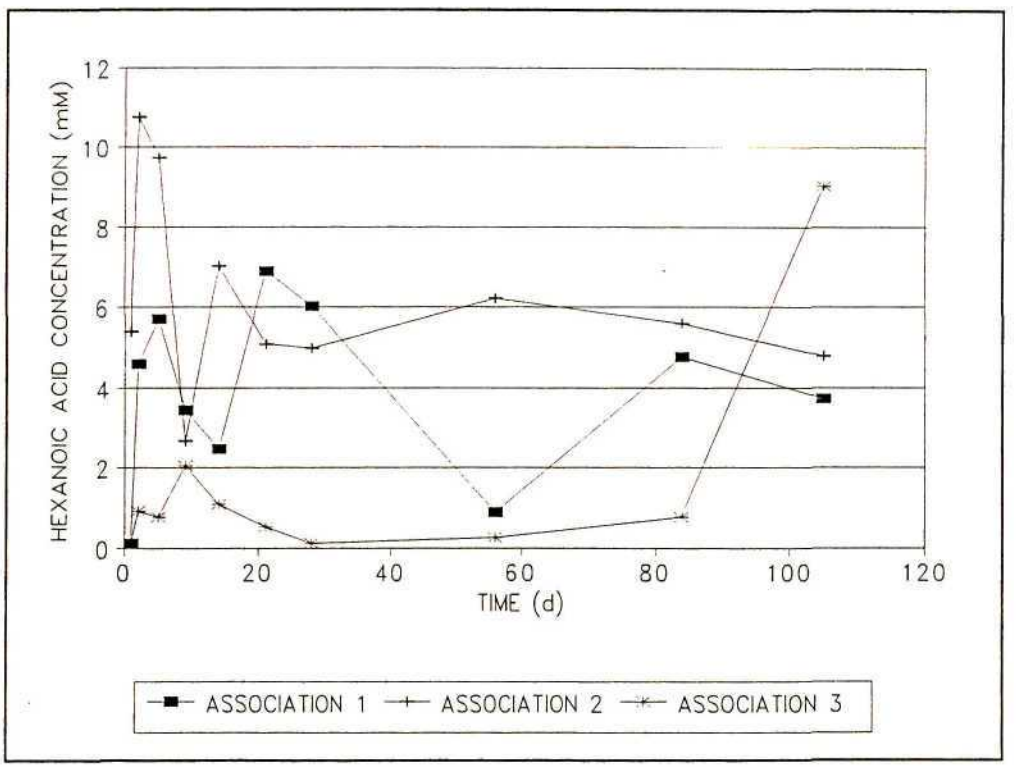


Figure 4.2. **Changes in residual hexanoic acid concentrations during aerobic CCU cultivation. The initial hexanoic acid concentration was 50mM (D = 0.1h^{-1}). The associations were enriched/isolated from: One. Landfill soil cover; Two. Soil from the landfill base liner where gas and leachate were actively emitted; and Three. Municipal refuse.**

ii. Turbidity

Figure 4.3 shows the turbidity readings of the aerobic CCUs, in which up to day 56 the highest turbidity readings corresponded to the lowest residual substate concentrations.

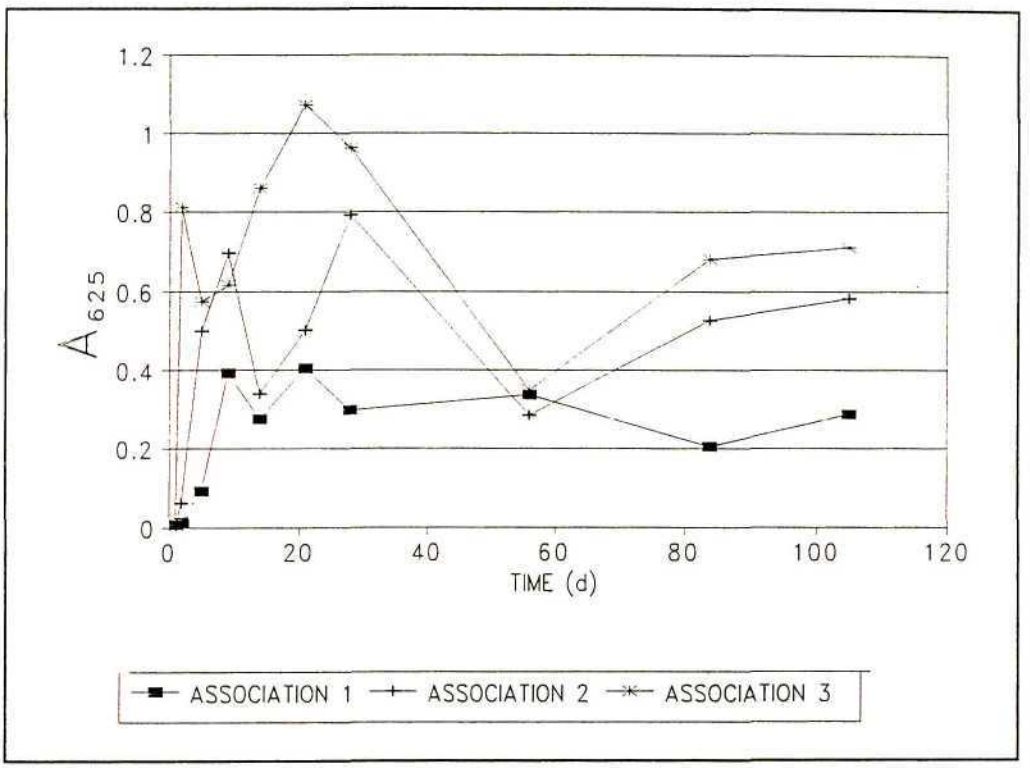


Figure 4.3. Changes in turbidity (A_{625}) during aerobic CCU cultivation (D = 0.1h^{-1}) of associations enriched/isolated from: One. Landfill soil cover; Two. Soil from the landfill base liner where gas and leachate were actively emitted; and Three. Municipal refuse.

iii. Image Analysis

Figure 4.4 shows the number of microorganisms.field^{-1}, as determined by image analysis, in the effluents of the CCUs. As with the OD determinations, Association three recorded the highest number of microorganisms when compared to Associations one and two.

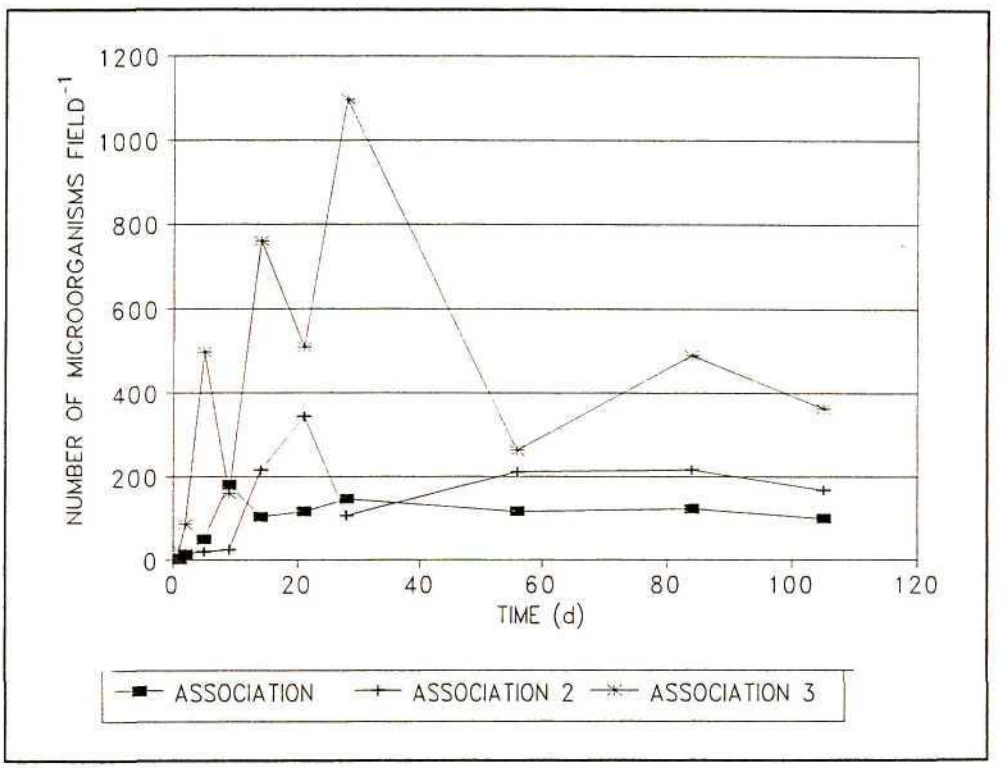


Figure 4.4. Changes in mean number of microorganisms in CCU effluents (D = 0.1h^{-1}) as determined by image analysis of fluorescent stained preparations (2.1.3). The numbers are quoted as means.field^{-1} of view (400X magnification). The three associations used were enriched/isolated from: One. Landfill soil cover; Two. Soil from the landfill base liner where gas and leachate were actively emitted; and Three. Municipal refuse.

iv. Plate Counts

Table 4.3 shows the number of colony-forming units present, on mineral salts agar supplemented with 10mM hexanoic acid (2.1.2), in the effluents of the CCUs. Plate counts monitor the number of viable cells and appeared, initially, to show conflicting results with large numbers apparent in some cultures in the early stages, followed by rapid decreases, and stable counts in others. However, after the first month of CCU operation relatively stable numbers were obtained for Associations one and two.

Table 4.3. Number of colony-forming units present in the influents and effluents of three aerobic CCUs. The colony-forming units were estimated on nutrient agar plates supplemented with 10mM hexanoic acid. The CCUs contained microbial associations enriched/isolated from: One. Landfill soil cover; two. Soil from the landfill base liner where gas and leachate were actively emitted; and three. Municipal refuse.

TIME (DAYS)	INFLUENT	CONTINUOUS CULTURE UNITS CULTURE EFFLUENT								
		ONE			TWO			THREE		
		DILUTION								
		10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}	10^{-6}	10^{-7}	10^{-8}
1	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
2	<30	>200	90	<30	<30	<30	<30	>200	57	<30
5	<30	>200	43	<30	<30	<30	0	>200	78	34
9	<30	150	<30	<30	103	<30	<30	>200	<30	<30
14	<30	126	<30	<30	37	<30	<30	>200	51	<30
21	<30	178	<30	<30	128	<30	<30	>200	42	<30
28	<30	82	<30	<30	46	<30	<30	>200	<30	<30
56	<30	145	<30	<30	38	<30	<30	154	<30	<30
84	<30	138	<30	<30	79	<30	<30	>200	<30	<30
105	<30	189	<30	<30	56	<30	<30	176	<30	<30

4.4 *DISCUSSION*

4.4.1 **Enrichment and Isolation**

To obtain microbial associations from landfill environments for use in subsequent experiments necessitated an enrichment/isolation programme. Free-living associations developed as the enrichment programme proceeded. These planktonic associations were then cultured in CCUs and were used in subsequent experiments. This ensured that uniform inocula were used at all times.

Enrichment cultures do not provide estimates of the abundance of a particular bacterial species in a given habitat or an indication of whether or not it is physiologically active in that environment (Parkes, 1982). They effect the enrichment of those bacteria which are best adapted to the selective conditions and thus outgrow their competitors. Interpretation of the roles played by particular bacteria in natural environments, when isolated following enrichment, should, therefore, be treated with great caution (Herbert, 1982). The enrichments and isolations were carried out with the knowledge that the physico-chemistry of soil components and refuse components were not investigated. No isolations of the component species of the associations were undertaken as the objective was to obtain associations for subsequent experiments involving surfaces.

During the enrichment programme the inocula were challenged with different concentrations of a common landfill intermediate. From the rates at which the substrate was catabolized conclusions were drawn on which concentration of hexanoic acid facilitated the fastest enrichment. The three sources of inoculum (soil cover, soil from the landfill base liner and municipal refuse) were compared for their ability to provide associations which catabolized the substrate rapidly.

Covering Soil: The results of the initial batch culture enrichments confirmed that the soil used contained microbial associations capable of catabolizing hexanoic acid concentrations < 50mM. Thus, inoculation of landfill covering soil with catabolic associations to attenuate vertically-migrating hexanoic acid would not be required.

The associations which degraded the molecule after the shortest lag period developed from an enrichment protocol of increased acid concentration with time. By exposing the landfill top soil microorganisms to a progressive increase in hexanoic acid concentration, as happens with a landfill site as the fermentation proceeds, one could, hopefully, ensure that catabolic populations developed.

Refuse: In the refuse mass high concentrations (50mM) of hexanoic acid can be aerobically catabolized. However, the experiment showed that aerobic catabolizm may only occur in freshly placed refuse or where oxygen is non-limiting. If the hexanoic acid is degraded in the refuse mass a reduced concentration would reach the soil cover thus reducing its phytotoxicity.

Soil Liner: The microbial complement of the soil liner effectively degraded low concentrations of hexanoic acid both in the presence and absence of oxygen. With 50mM hexanoic acid the aerobic cultures took longer (90% of the substrate degraded after 20d) than the other concentrations. With 50mM hexanoic acid the anaerobic cultures appeared inhibited after 30d (< 20% catabolized).

Comparison of Aerobic and Anaerobic Batch Cultures: When the results of the aerobic and anaerobic enrichments were compared, it appeared that the aerobic degradation of hexanoic acid occurred at higher rates, and with higher initial concentrations, without any noticeable inhibitory affects, than the anaerobic equivalents. This could, therefore, prove beneficial to the plants in the aerobic covering soil.

Atlas, Horowitz, Krichevsky and Bej (1991) found that taxonomic and genetic diversities of microbial communities challenged by chemical pollutants were lower in undisturbed reference communities than in disturbed communities. The dominant populations had enhanced physiological tolerances and substrate utilization capabilities which indicated that physiological versatility is an adaptive characteristic of populations which successfully compete within disturbed communities (Atlas *et al.*, 1991).

4.4.2 Continuous Culture

The subsequent experiments which focused on surface-attached microbial associations required uniform inocula. Therefore, after enrichment, the isolated associations were maintained in aerobic or anaerobic CCUs (see section 4.2.6). The CCU selects for free-living associations in preference to surface-attached associations. This suggests that it may not be appropriate to use CCUs in experiments involving surface-attached associations. Continuous culture was used to control the physiological conditions and maintain the isolated microbial associations over long periods.

Enumeration Methods: The different enumeration methods used gave similar results but image analysis proved the fastest. Residual substrate determinations using gas chromatography techniques, IA (Figure 4.4) and plate counts (Table 4.3) were made on each influent reservoir when it was almost empty. Contamination occasionally occurred due to microbial backgrowth. When this happened a new influent reservoir was immediately attached to the CCUs. Use of the image analyzer to count large numbers of microorganisms was useful but was even more beneficial to scan large areas of a slide where there were no, or few, microorganisms. The image analyzer is particularly effective for this routine but essential task (Dudley, Wallis, Bruton, and Senior, 1992c). The traditional method of plate counts (Table 4.3) identified the presence of contaminants but only after a finite (five d) incubation period.

Anaerobic Continuous Culture Units: The results of turbidity, IA, GC residual substrate determinations and dilution plate counts of the anaerobic CCUs are not shown. Due to numerous losses in nitrogen pressure, which led to oxygen ingression, the anaerobic cultures had to be reinoculated but never developed satisfactorily for further study. Also, due to their higher specific growth rates, the aerobic microbial associations were used in the subsequent experimental programme.

CHAPTER FIVE

5. INVESTIGATIONS OF AN ENRICHED, UNDEFINED LANDFILL MICROBIAL ASSOCIATION IN CONTINUOUS CULTURE MICROSCOPY UNITS: PRIMARY ADHESION AND BIOFILM DEVELOPMENT

5.1 *INTRODUCTION*

5.1.1 **Biofilms**

The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic forces and the dominant microbial components of the biofilm (Lappin-Scott *et al.*, 1993; Bos, Mei, Busscher and van der Mei, 1995). On surfaces, biofilms invariably develop and consist of a range of microorganisms, including bacteria, fungi, protozoa and, possibly, viruses and extracellular products (Ellwood, Keevil, Marsh and Wadell, 1982; Cowan, Warren and Fletcher, 1991). The biofilm may serve to protect the microorganisms from inimical influences such as substance toxicity (Keevil, Dowsett and Rodgers, 1993).

Responses of microorganisms to physical changes are highly suitable biological indicators of microorganism status. Dominance of indicator species with distinctive morphologies allows quantification of responses by epifluorescence microscopy and image analysis of undisturbed, unstained communities (Wynn-Williams, 1996).

During the early stages of colonization microcolonies are observed primarily in crevices and other regions shelted from hydraulic shear forces (Massol-Deyà *et al.*, 1995). Nutrient conditions in the liquid phase influence the growth of suspended and attached organisms, as well as cell movement to the solid-liquid interface and the rate of attachment onto the substratum. Hence, the diffusive, advective and hydrodynamic properties of a specific system cannot be neglected in order to understand and simulate microbial colonization (Mueller, 1996).

5.1.2 **Microbial Association**

A major determinant of species diversity in biofilms is the presence of inorganic and organic surfaces for microbial attachment and growth (Cowan *et al.*, 1991). Development of microbial biofilms on these surfaces is often accompanied by the production of mucilaginous exudates which, in turn, limit the diffusion of both extracellular enzymes and their products thus facilitating the retention of microbial association integrity. This is essential for efficient metabolism under anoxic conditions since no single species can effect the mineralization of a macromolecule (Senior, 1991). Mixed culture studies are particularly important in investigating species which fail to colonize surfaces unless other primary colonizers are already established (Wolfaardt *et al.*, 1994). To examine the considerable number of variables which influence growth on surfaces Caldwell and Lawrence (1989) used single associations cultured under defined, controlled conditions in CCMUs. It was recognized that this work must ultimately be extended to consider interactions between discrete, but spatially separated, microbial species/associations (Cowan *et al.*, 1991).

There is limited literature describing the quantification of mixed culture acclimatization (Smith, Nickels, Kerger, Davis, Collins, Wilson, McNabb and White, 1986; Escher and Charachlis, 1988) especially in a kinetic context (Beeman and Suflita, 1987; Brown and Gilbert, 1993). Species interactions and co-metabolism, which can render a refractory compound biodegradable, are extremely important phenomena (Caldwell and Germida, 1985; Cowan *et al,*. 1991). Unfortunately, the acclimatization of mixed cultures to single and mixed substrates is poorly understood since mixed culture/mixed substrate systems are not modeled accurately by monoculture/single substrate systems (Evans and Ahlert, 1987).

5.1.3 **Continuous Culture Microscopy Units**

One of the biggest problems facing the microbial ecologist is sampling (Wimpenny, Lovitt and Coombs, 1983; Downes *et al.*, 1995). Any model film-fermenter must have facilities to enable discrete, representative and reproducible samples to be taken. When choosing a model system for *in vitro* biofilm study, it is essential to select one that closely mimics the *in vivo* environment (Thomas and Paul, 1996). A realistic approach is to study the

microorganism(s) in a Continuous Culture Microscopy Unit (CCMU) where it/they can be examined without disruption (Jones *et al.*, 1992b; Addy, Slayne and Wade, 1993; Nivens *et al.*, 1995).

Continuous Culture Microscopy Units are also one of the most convenient systems for the study of surface microenvironments under defined conditions (Kjelleberg, Humphrey and Marshall, 1982). Continuous-flow slide cultivation not only offers the possibility of studying living cells within surface microenvironments but also provides opportunity to study the behaviour of individual cells under defined conditions. Biofilm investigation *per se* is, however, insufficient and must be complemented by other experiments which involve traditional destructive sampling such as scanning or transmission electron microscopy.

5.1.4 **Fluid Parameters**

The fluid parameters which may affect biofilm formation need to be investigated and strictly controlled (Mueller, 1996). For a model such as the CCMU (or Flow Cell) to be useful, all parameters must be controllable. Any chosen regime of nutrient concentration, dilution rate, shear rate and temperature must be easy to set and maintain (Berg and Block, 1984). It must also be easy to achieve and maintain sterility so that monocultures or preselected biofilm communities may be investigated without contaminants. If it is necessary to know the exchange properties of a CCMU the cell should be calibrated with a dye which has a diffusion coefficient roughly equal to the substrate of interest under the conditions of the planned experiments (Berg and Block, 1984).

Kober, Lawrence, Sutton and Caldwell (1989) used computer-enhanced microscopy (CEM) to monitor bacteria colonizing the inner surface of a CCMU cell. Image analysis provides a rapid and reliable means of measuring microcolony number and area and cell motility. During continuous-flow slide culture, neither the rate of growth nor the timing of recolonization were influenced by the flow rate or microbial motility. However, the degree of reattachment of recolonizing cells was both flow and motility dependent.

The deposition efficiency and reversibility of bacterial adhesion on different substratum surfaces under flow have been determined by real-time *in situ* image analysis (Meinders *et al.*, 1995). These authors concluded that initial bacterial adhesion could be explained in terms of overall physico-chemical surface properties and that it is mediated by reversible, secondary minimum DLVO (Lifshitz-Van der Waals and electrostatic) interactions.

5.1.5 **Image Analysis**

Previous studies of population dynamics within surface microenvironments have been limited by a lack of analytical methodology for estimating the growth of individual cells and microcolonies (Harvey and Young, 1980; White, 1986). To determine the extent to which the growth rate-independent microorganisms are involved, the communities must be examined in relatively undisturbed tracts. Unfortunately, the heterogeneity and complexity of the biofilm-forming associations renders quantification of colonization and changes in response to, for example, leachate perturbations, difficult (Wilson and McNabb, 1983; Smith *et al.*, 1986). These difficulties can, however, be overcome by the use of image analysis (Wynn-Williams, 1988; Dudley *et al.*, 1994b; Busscher and Van Der Mei, 1995).

5.1.6 **Objectives**

The objectives of this part of the investigation were to examine primary adhesion as well as biofilm development, on glass surfaces, of an undefined aerobic microbial association enriched/isolated from municipal refuse (Inoculum three) (4.2.1/5). The study was designed to determine if it was possible to use image analysis to study and classify component species in landfill microbial associations thereby allowing interpretation of components of the ecosystem.

To ensure accurate measurements, the precision of the image analyzer had first to be established. To use grey-level measurements in image analysis a standard curve of increasing light intensity is required and this had first to be constructed (Appendix Six, p135). Before the CCMUs were used their exchange properties had to be calibrated. This was done with bromophenol blue dye.

The effects of increasing the dilution rate and substrate concentration on the growth of the association in CCMUs had also to be examined. To ensure that the different component species of the association were able to multiply under the defined conditions a detailed investigation of the component species was necessary. The optimum culture conditions for microbial multiplication had then to be selected.

5.2 *MATERIALS AND METHODS*

5.2.1 Accuracy Evaluation of Small Objects

A test was undertaken to determine if the image analysis programme could distinguish the smallest microorganisms at the highest microscope magnification employed (1000X). Ball bearings (total = 330) representing small cocci were used due to their similar shape and suitability for evaluating the measurement of small objects. The ball bearing's diameter was 1.5mm (< 10% error) (Bearings International, Pietermaritzburg). An image of the ball bearings was captured so that when it appeared on the image analyzer screen it was the same size as the smallest coccus (> 71 pixels; equivalent to < 0.02μm^2) (Dudley, Howgrave-Graham, Isherwood and Senior, 1993b). A light table (Mechanical Instrument Workshop, University of Natal, Pietermarizburg) was used as the background to the object. The perimeter, area and diameter of circle (DCIRCLE) of the ball bearing image were measured.

5.2.2 Continuous Culture Microscopy Unit

The exchange properties of the CCMUs were measured by drawing solutions of the dye bromophenol blue (MW = 600) through the unit with a peristaltic pump (Ismatec IPN 24B) (Berg and Block, 1984). A CCMU was set up and run at a dilution rate of 0.5h^{-1} (0.0425ml h^{-1}) which equated to a laminar flow velocity of 4.72x10^{-4}ml s^{-1} (1.7ml h^{-1}). The integrated optical density (OPTDI) at the centre of each CCMU was recorded on video tape at the lowest microscope magnification (25X). Images were captured from the video tape and analyzed by image analysis (see section 3.3.2).

5.2.3 Continuous Culture Microscopy Unit Operation Parameters for Dilution and Substrate Concentration Experiments

The CCMUs were inoculated (0.085ml) with supernatant from the Continuous Culture Unit (CCU). Of the three associations (landfill soil cover, soil from the landfill base liner and municipal refuse) only the association (CCU three) isolated from municipal refuse (Inoculum three) (see section 4.2.1/5) was used in this experiment. Duplicate CCMUs were used for the dilution rate experiment (see section 5.3.3) while triplicate CCMUs were used for the substrate concentration experiment (see section 5.3.4). In each case only one unit was analyzed. For the dilution rate experiment the peristaltic pump (Ismatec IPN 24B) was started 12h after inoculation while for the substrate concentration experiment 24h were allowed to pass before initiating flow. For the former, the CCMUs were sampled and analyzed 24, 26, 28, 60 and 72h after inoculation while for the latter analyses were made 36, 48, 60, 72 and 94h after inoculation. The microscope magnification was 400X (equivalent to 4920μm^2 on the image analysis screen, calculated by sizing objects of known dimensions on the screen). For each CCMU the numbers of individual microorganisms.field^{-1} were counted, the area colonized was calculated and the mean optical density was determined by image analysis.

5.2.4 Detailed Investigation of Component Species

After 60h of operation at a dilution rate of 0.5h^{-1} and a hexanoic acid concentration of 50mM, a CCMU from the substrate concentration experiment was destructively analyzed. A Zeiss Axiophot microscope equipped for fluorescence microscopy was used at a magnification of 1000X which was equivalent to 7300X (45.4834μm^2) on the image analysis screen (Dudley *et al.*, 1992b). The CCMU was prepared by standard methods for fluorescence microscopy (Trolldenier, 1973). For assessing biofilm development, a continuous field (X axis) at a specific Y axis point was captured on video tape. The average area (μm^2) of each microbial morphotype, the circularity (fcircle), elongation and number (Kontron Elektronik, 1991) of microbial cells were determined by image analysis.

5.2.5 Microbial Colonization of a Continuous Culture Microscopy Unit fed 10mM Hexanoic Acid at a Dilution Rate of $0.5h^{-1}$

Continuous culture microscope units (CCMUs) were set up and operated at a dilution rate of $0.5h^{-1}$ ($0.0425ml\ h^{-1}$) which equated to a laminar flow velocity of $0.000472ml\ s^{-1}$ ($1.7ml\ h^{-1}$). The medium was mineral salts (see section 4.2.2) supplemented with 10mM hexanoic acid (see section 4.2.4) as the limiting carbon source (final concentration). The CCMUs were individually inoculated ($10\%v.v^{-1}$) with supernatant from CCU three. The CCMUs were operated in batch mode for 24h prior to continuous cultivation. At recorded times (24h, then every 12h, until 132h) the CCMUs were removed and analyzed for growth (see section 4.2.7) until determinations could no longer be made due to biofilm thickness.

Once the growth of the microorganisms had been recorded image analysis was made. The microorganisms were monitored by: increases in number of cells attached to the surface; increases in the size (area) of the attached microorganisms (either as individual cells if possible or as an area occupied by the attached population of cells); elongations (length) and fcircles; and increases in densities (grey-level value) of the cells. The standard curve of increasing light intensity is shown in Appendix Six (p135).

5.3 *EXPERIMENTAL RESULTS*

5.3.1 Accuracy Evaluation of Small Objects

The image analyzer tended to overestimate the size (DCIRCLE) of the ball bearings by 7.5% (counts 330; lower bound 1.48; upper bound 1.83; variance 0.055; standard deviation 0.235). The light table used to provide contrast to the objects resulted in shadows which may have contributed to the increased apparent size of the objects. This is an acceptable error level (variance 0.055; standard deviation 0.235) as trends in the results produced by the image analyser of the different test procedures could still be clearly shown. Bloem *et al.* (1995) found that estimates of cell volumes (determined from surface area) were reliable although absolute values were affected by the settings for detector sensitivity.

5.3.2 Continuous Culture Microscopy Unit Calibration

The time change in optical density of the dye is shown in Figure 5.1. Exchange of the dye was complete within four seconds at a dilution rate of $0.5h^{-1}$.

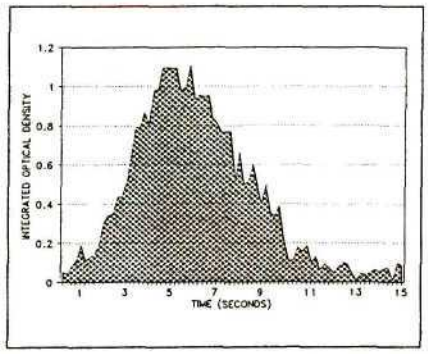


Figure 5.1. Time study of the change in integrated optical density at the centre of the continuous culture microscopy unit. Bromophenol blue dye was used (D = $0.5h^{-1}$).

5.3.3 Dilution Rate

The purpose of this part of the experiment was to determine which dilution rate was optimal for growth of the association. Therefore the magnification was low (400X) making the probability of selecting other fields and obtaining similar results high. At this low magnification accurate measurements could not be obtained so only area colonized and numbers of microorganisms.field^{-1} were selected. The homogeneity of the biofilm was not investigated at this stage of the experiment.

At the dilution rates (D) used, viz. $0.1h^{-1}$, $0.2h^{-1}$, $0.5h^{-1}$, $1.0h^{-1}$ and $2.0h^{-1}$, the equivalent laminar flows were: $9.44x10^{-5}$, $1.89x10^{-4}$, $4.72x10^{-4}$, $9.44x10^{-4}$ and $1.89x10^{-3}$ml s^{-1}. The irrigation medium was 10mM hexanoic acid-supplemented mineral salts solution (4.2.2). The number of cells.field^{-1}, optical density and colonized area (μm^2) of microbial biofilms cultured at various dilution rates are presented below.

At D = $0.1h^{-1}$ a marked increase in cell number (19.4 microorganisms.field^{-1} at 24h to over 175.1 at 36h) resulted. Thereafter, negligible fluctuation in cell number.field^{-1} occurred (162 microorganisms.field^{-1} at 72h). A progressive increase in the optical density.field^{-1} (0.239 at 48h compared to 0.447 at 72h) contrasted with a slow reduction in the average colonized area.field^{-1} ($0.159\mu m^2$ at 36h) until 60h ($0.100\mu m^2$) when the colonized area increased ($0.199\mu m^2$ at 72h). The biofilm then appeared to increase in thickness (greater

optical density.field^{-1}) but not in cell number or area colonized.field^{-1}, possibly due to an elevated amount of extracellar material. No further details of the cause of the increase in optical density were investigated as this was beyond the scope of the current investigation.

At D = 0.2h^{-1} the optical density rapidly increased (0.1 to 0.7) between 24h and 36h and then remained relatively constant (0.657 at 72h). This was a substantial increase from the lower dilution rate. The average colonized area.field^{-1} did not increase between 24h (0.083μm^2) and 60h (0.065μm^2) but thereafter increased significantly (0.141μm^2 at 72h) while the number of microorganisms.field^{-1} actually decreased (945 at 36h versus 112.5 at 72h). No cell multiplication was recorded from 36h onwards.

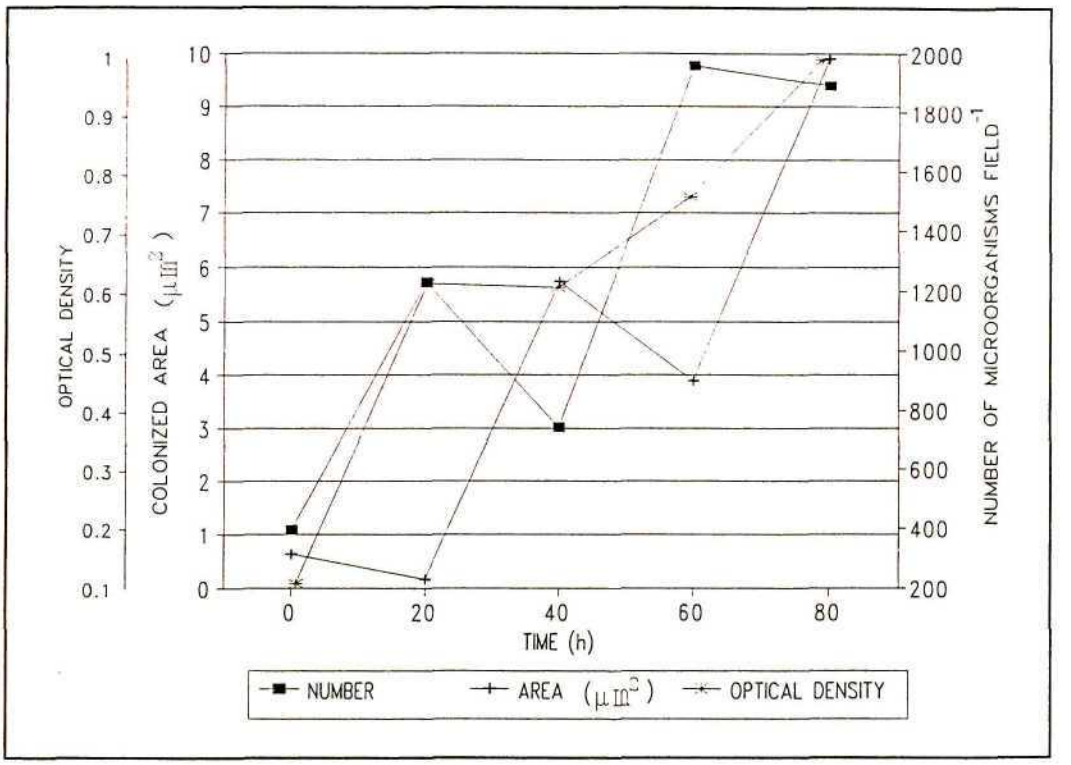


Figure 5.2. **Changes in the number of cells.field^{-1}, colonized area (μm^2) and optical density with time of a microbial association cultured on hexanoic acid (10mM) - supplemented mineral salts medium (D = 0.5h^{-1}). The CCMU was inoculated with an association enriched/isolated from municipal refuse (Inoculum three).**

Figure 5.2 shows the number of cells.field^{-1}, colonized area (μm^2) and optical density of a microbial association cultured at a dilution rate of 0.5h^{-1}. The highest biofilm growth rate occurred at this dilution rate. All the monitored parameters increased substantially. The number of microorganisms.field^{-1} was over 1800 from 60h onwards, while the area colonized.field^{-1} (9.888μm^2 at 72h) was substantially greater than it was at the lower dilutions. The optical density increased to a maximum of 0.998 at 72h.

At D = 1.0h^{-1} and 2.0h^{-1} the numbers of microorganisms were much lower than at the lower dilution rates and washout occurred. With a dilution rate of 1.0h^{-1} the maximum optical density was 0.09 (at 60h) and maximum number of microorganisms.field^{-1} was 28.8 (at 48h and 60h) while the maximum area colonized.field^{-1} was 0.34μm^2 at 72h. At D = 2.0h^{-1} the monitored parameters increased with time until the experiment was terminated at 72h but the final values were all lower than those obtained at a dilution rate of 1.0h^{-1}.

5.3.4 **Substrate Concentration**

The hexanoic acid concentrations used were 0, 5, 10, 25 and 50mM. The dilution rate used (0.5h^{-1}) was the optimum identified in the dilution rate experiment (5.3.3).

With unsupplemented mineral salts solution the optical density decreased with time (0.25 at 36h to 0.079 at 94h). The number of microorganisms initially decreased (162.field^{-1} at 36h to 80.field^{-1} at 60h) then increased (120.field^{-1} at 94h). Over the 36h-48h period the area colonized by the microorganisms tripled (from 0.16μm^2 at 36h to 0.45μm^2 at 48h) then decreased with time (0.2μm^2 at 94h).

With an influent substrate concentration of 5mM hexanoic acid the area colonized by the microorganisms increased slowly (0.8μm^2.field^{-1} at 48h to 0.90μm^2.field^{-1} at 60h) as did the optical density (< 0.6 at 36h to 0.66 at 94h). The cell number.field^{-1} initially decreased until 72h (760 at 36h to 220 at 72h), probably due to washout of non-attached cells, but then began to increase (442 microorganisms.field^{-1} at 94h). These results were similar to those of the control where, possibly, initial attachment of the association was followed by detachment.

With a substrate concentration of 10mM hexanoic acid the optical density increased (0.65 at 36h to 0.78 at 48h), then decreased (0.66 at 60h) and, finally, increased again (0.74 at 94h). There was minimal increase in the area colonized by the microbial association until 60h (6.14μm^2). Although the cell number.field^{-1} increased slowly the total was lower (198 cells.field^{-1} at 94h) than with 5mM hexanoic acid at corresponding times.

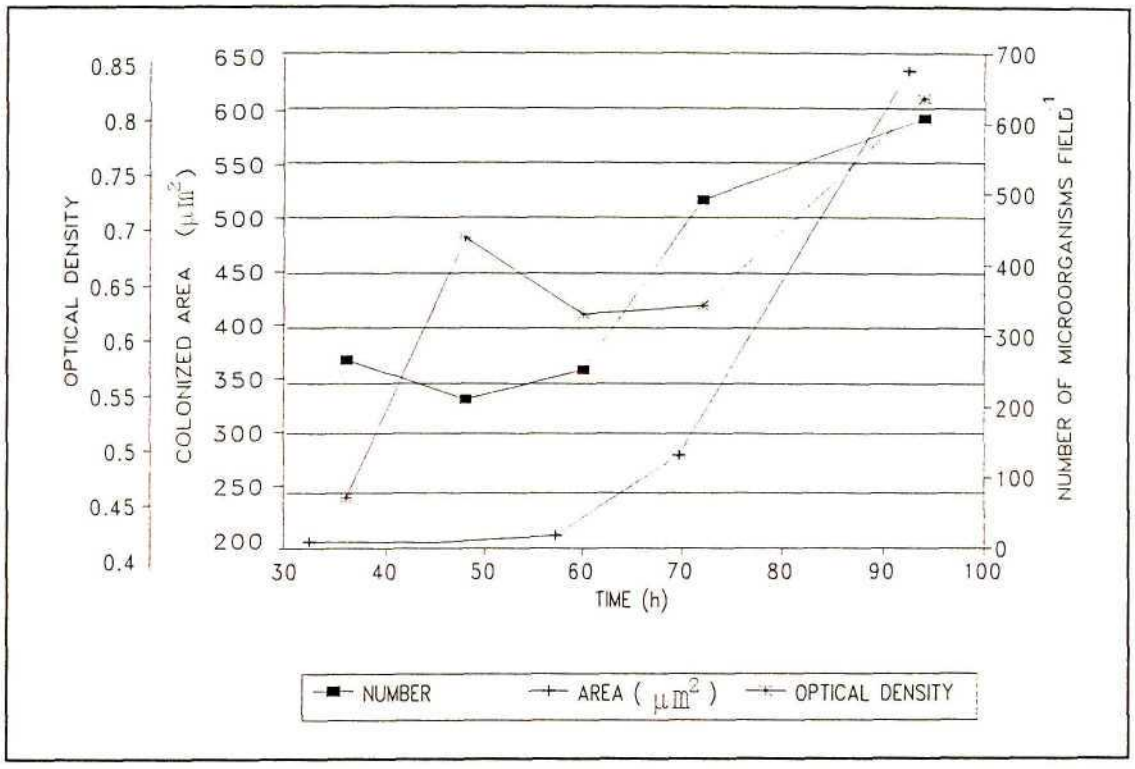


Figure 5.3 Changes in the number of cells.field^{-1}, colonized area (μm^2) and optical density with time of a microbial association cultured on mineral salts medium supplemented with 50mM hexanoic acid (D = 0.5h^{-1}). The CCMU was inoculated with an association enriched/isolated from municipal refuse.

With 25mM hexanoic acid both the optical density and microbial number.field^{-1} decreased over the first 48h of the experiment. Thereafter, however the values increased 0.49 at 48h to 0.85 at 94h and 46 at 48h to 553 at 94h respectively. The microbially colonized area was higher during this experiment (16.1μm^2.field^{-1} at 94h) than it was with lower concentrations of hexanoic acid. With 50mM hexanoic acid all the measured parameters increased substantially with time (608 cells.field^{-1}, 72μm^2 colonized.field^{-1} and 0.8 optical density at 94h) (Figure 5.3). Bloem *et al.* (1995) similarly found significant increases in

the values of the cell parameters (numbers, cell volumes) following supplementation with nutrients.

5.3.5 **Detailed Investigation of Component Species**

Biofilm quantification of the rods, cocci, fungal hyphae and fungal spores is shown in Figure 5.4 and Tables 5.1 and 5.2. For assessing biofilm development no replicates were analysed as a continuous field was captured. This could be thought of as equivalent to 3 duplicates per image set if 36 sets of images had been captured (108 images). Both rods and cocci were distributed throughout the CCMU. The less numerous fungal hyphae were mainly concentrated near the effluent port (Image Numbers 80 - 108, Figure 5.4) of the CCMU.

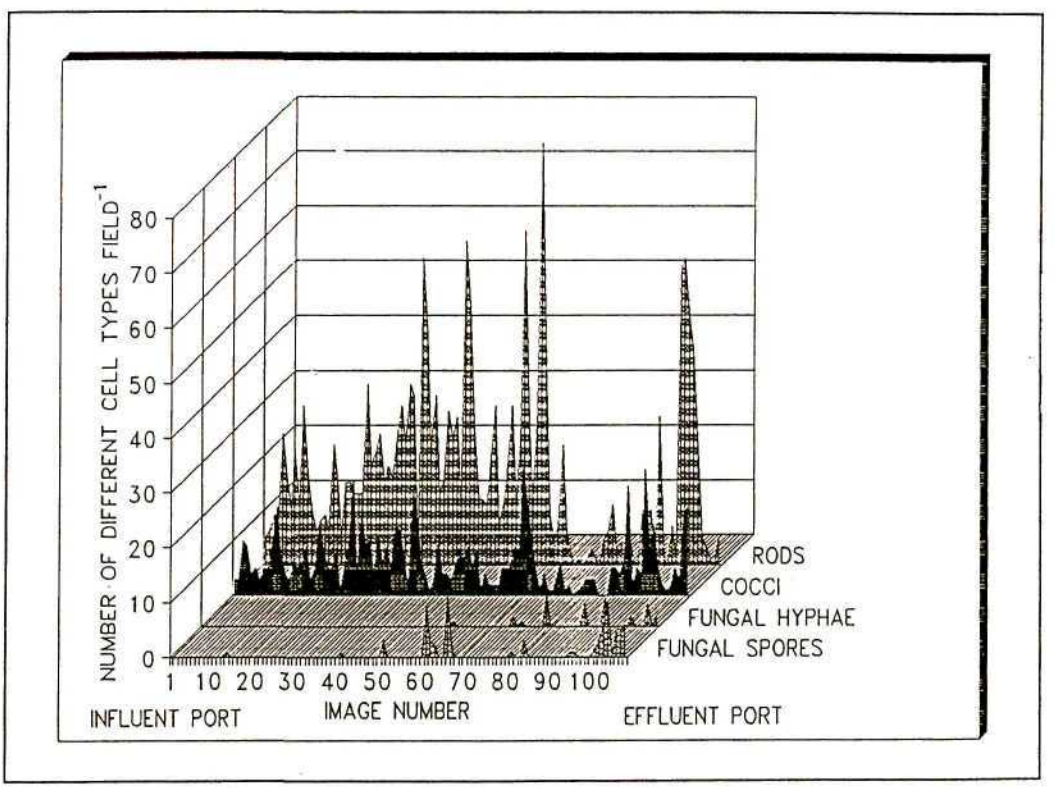


Figure 5.4. Quantification of rods, cocci, fungal hyphae and fungal spores in a microbial association, isolated from municipal refuse, (Inoculum three) cultured in a CCMU. The component types were identified by image analysis (3.3.2) and the images were captured after 60h of operation (4.2.7). The hexanoic acid concentration was 50mM (D = 0.5h^{-1}).

*Table 5.1. Total and average cell number.field^{-1} of the components of a mic. association enriched/isolated from municipal refuse (Inoculum three). A. 60h of operation 108 images were captured (see section 4.2.7) from th CCMU. The hexanoic acid concentration was 50mM (D = 0.5h^{-1}).

	RODS	COCCI	FUNGAL HYPHAE	FUNGAL SPORES
TOTAL NUMBER	4724	692	27.0	74.0
AVERAGE NUMBER.FIELD^{-1}	43.74	6.40	0.25	0.69

*Table 5.2. Variation, standard deviation and mean of the measured parameters: area, circularity (fcircle) and elongation of the components of a microbial association isolated from municipal refuse (Inoculum three). After 60h of operation 108 images were captured from the CCMU. For assessing biofilm development a continuous field (X axis) at a specific Y axis point was captured on video tape. The hexanoic acid concentration was 50mM (D = 0.5h^{-1}). Fcircle = perimeter^2.(4.π.area)$^{-1}$ and Elongation = largest Feret diameter/shortest Feret diameter.

		VARIATION	STANDARD DEVIATION	MEAN
AREA (μm^2)	RODS	0.0005	0.02	0.03
	COCCI	0.0003	0.02	0.02
	HYPHAE	0.2000	0.45	0.65
	SPORES	0.8500	0.92	1.86
FCIRCLE	RODS	0.0130	0.12	0.34
	COCCI	0.0140	0.12	0.69
	HYPHAE	0.0150	0.12	0.08
	SPORES	0.0400	0.20	0.62
ELONGATION	RODS	0.3100	0.56	2.03
	COCCI	0.0200	0.13	1.31
	HYPHAE	344	18.5	29.5
	SPORES	0.1200	0.35	1.48

*For assessing biofilm development no replicates were analysed as a continuous field was captured. This could be thought of as equivalent to 3 duplicates per image set if 36 sets of images had been captured (108 images)

5.3.6 Microbial Colonization of a Continuous Culture Microscopy Unit fed 10mM Hexanoic Acid at a Dilution Rate of $0.5h^{-1}$

The numbers and areas of microorganisms which attached to the surface with time were determined from successive images of the same field of view. The total surface area occupied by rods, cocci and fungal hyphae increased throughout the experiment (Figure 5.5). This was expected as microbial colonization of the CCMU was proceeding.

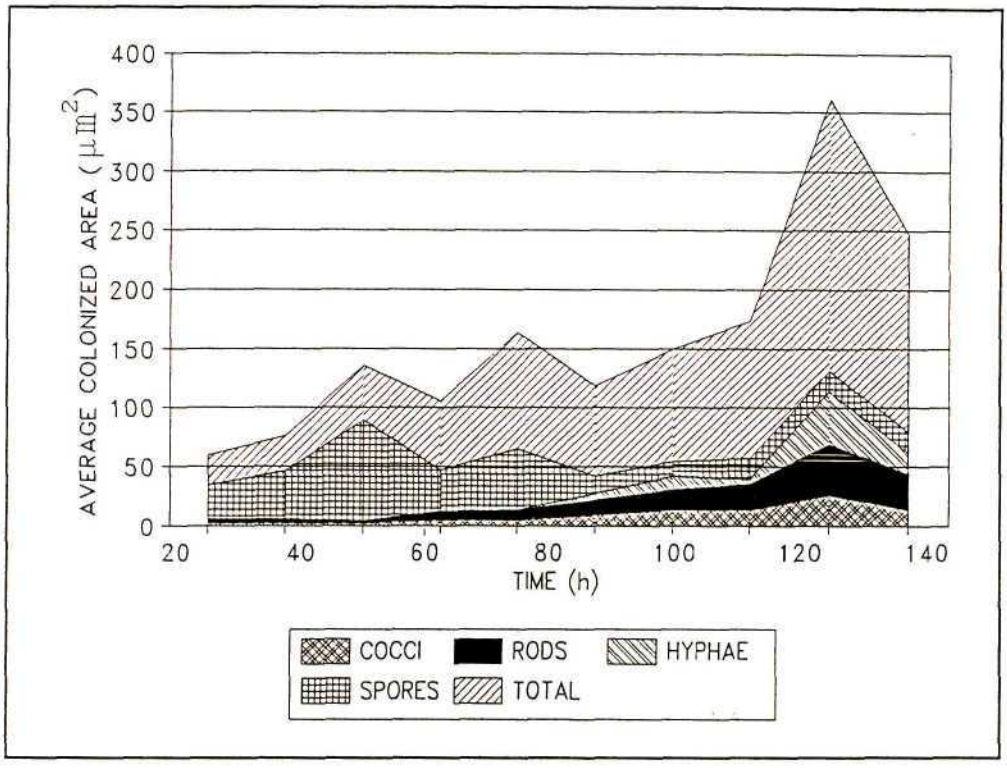


Figure 5.5. Changes in average area colonized by rods, cocci, fungal hyphae, fungal spores and total microbial surface area.field^{-1} in a CCMU with time. The images were captured (4.2.7) and the component types identified by image analysis (3.3.2). Hexanoic acid concentration = 10mM (D = $0.5h^{-1}$). The inoculum was enriched/isolated from municipal refuse (Inoculum three).

After initial attachment the total area occupied by fungal spores decreased due to germination or washout. The average colonized area (determined from the field-based

measurements) of microorganisms (Figure 5.5) increased up to 120h then appeared to decrease (132h) due to thick biofilm development which made it difficult to distinguish individual microorganisms and morphological categories. The average colonized area was calculated from all objects in the field of view resulted in an area > the sum of the components. This was accompanied by reductions in the percentage of discriminated phase (3.1) (the objects measured) and colonized area (total field) of microorganisms at 132h (from 100% and 225μm^2 at 120h to < 80% and 175μm^2 at 132h) (Figure 5.6). The area of the discriminated phase increased from 12μm^2 to > 225μm^2 while the percentage of the discriminated phase rose from approximately 20% to 100%.

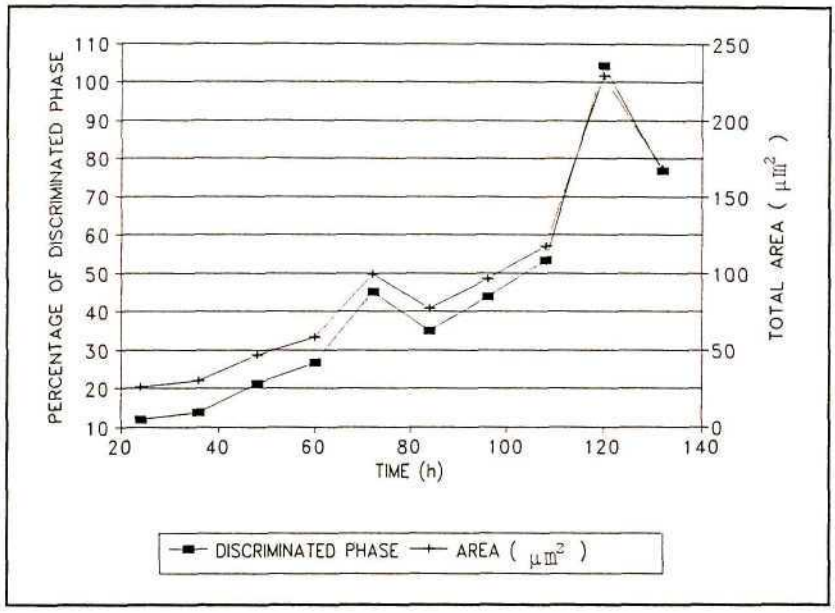


Figure 5.6. Changes in relative area of discriminated phase (%) compared to area (μm^2).field^{-1} of a microbial association cultured in a CCMU with time. The hexanoic acid concentration was 10mM (D = 0.5h^{-1}). The CCMU was inoculated with an association enriched/isolated from municipal refuse (Inoculum three).

At most sampling times the average rod surface area fluctuated between 0.125μm^2 and 0.145μm^2. The one exception was at 96h when the average surface area of the rods decreased substantially to 0.106μm^2. By contrast, the average elongation (length) of the

rods remained constant throughout the biofilm development. There was no discrimiation of cells at different stages in the cell cycle in this study which may prove illuminating.

Fungal hyphae grew substantially until 84h with increases in both elongation (length) (2.3.field^{-1} at 36h to 5.5.field^{-1} at 84h) and area (0.03μm^2.field^{-1} at 36h to 0.13μm^2.field^{-1} at 84h) (Figure 5.7) with an increase in number (from 1.field^{-1} at 36h to 10.field^{-1} at 84h). Thereafter, the elongation decreased (3.3.field^{-1} at 132h) while the average area of the hyphae decreased (0.06μm^2 at 108h) but then increased substantially (0.15μm^2 at 132h). The number of hyphae increased substantially during this period (from 15.field^{-1} at 108h to 84.field^{-1} at 120h).

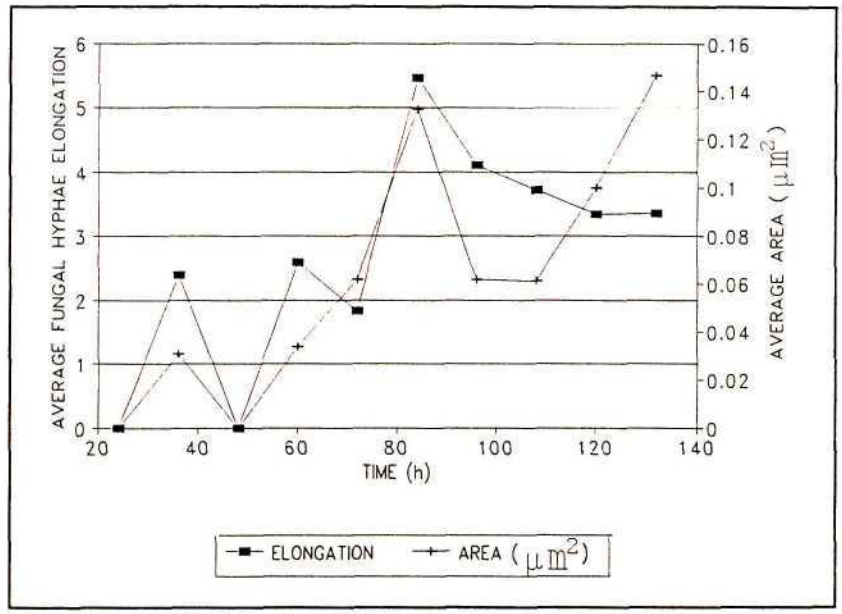


Figure 5.7. Changes in the average elongation (length) and area colonized by fungal hyphae.field^{-1}, in a CCMU with time. The hexanoic acid concentration was 10mM (D = 0.5h^{-1}). The CCMU was inoculated with an association enriched/isolated from municipal refuse (Inoculum three).

The circularity (fcircle) of the cocci changed little in the CCMU in the presence of 10mM hexanoic acid. This was not paralleled by the surface area measurements. When the number of cocci was compared to the average surface area a reduction in cell size before an increase in number appeared to occur. The average number of cocci.field^{-1} increased from 48h (100.field^{-1}) to > 1000 at 120h. The average surface area of the cocci appeared to decrease (to a minimum of 0.0198μm^2) before significant increases in numbers occurred, ultimately, reaching a maximum (0.022μm^2) at 96h. The average surface area of the cocci after 84h remained within the size range of 0.0215 - 0.0220μm^2.

The mean grey-level of the microorganisms increased (from 60 at 24h to < 250 at 132h) (Figure 5.8) and the mean transmission level (not shown) followed this trend. The integrated optical density followed a similar pattern with the exception that at 108h the optical density decreased substantially (Figure 5.8). The mean optical density (not shown) decreased similarly. The standard deviations of the grey-level, transmission and optical density measurements of the microorganisms are given in Table 5.3.

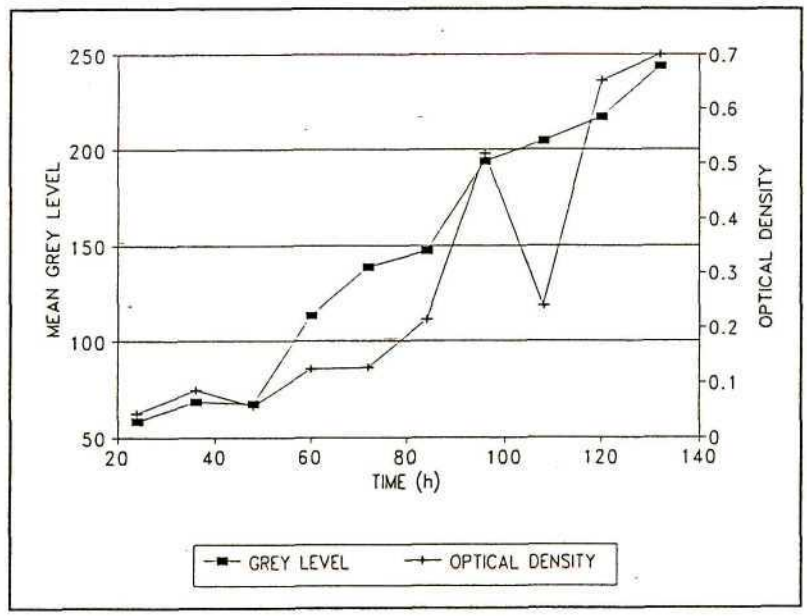


Figure 5.8. Changes in optical density measurements with time (mean grey-level and integrated optical density) of a microbial association isolated from municipal refuse (Inoculum three) cultured in a CCMU. The hexanoic acid concentration was 10 mM (D = 0.5h^{-1}).

Table 5.3. Standard deviations of grey-level and optical density measurements of microorganisms with time in a CCMU. The hexanoic acid concentration was 10mM (D = $0.5h^{-1}$). The CCMU was inoculated with an association enriched/isolated from municipal refuse (Inoculum three).

TIME (h)	STANDARD DEVIATION OF GREY-LEVEL (GREYSD)	STANDARD DEVIATION OF OPTICAL DENSITY (OPTDM)
24	3.386	0.044
36	5.093	0.064
48	4.276	0.046
60	3.684	0.046
72	3.237	0.045
84	3.975	0.050
96	4.413	0.052
108	4.014	0.041
120	3.078	0.043
132	3.789	0.049

5.4 *DISCUSSION*

In the present study, monitoring a microbial association by means of image analysis to identify the component types continued until the biofilm became impenetrable and prevented light microscope use. Such monitoring facilitates calculation of average circularity, elongation and area colonized by each component type in the association.

The accuracy of the image analyzer was determined for use in the evaluation of the small cocci present. It was found that it was not 100% accurate for small objects ($< 0.02\mu m^2$) although its accuracy ($\approx$7%) allowed qualitative comparisons between treatments to be made e.g., trends in very small objects were seen, not actual individual measurements. If a higher magnification or camera with greater resolution had been used the precision would have improved. Image analysis in this study provided useful information for initial biofilm quantification.

This study was aimed primarily at method development. Glass, although not normally a material found in landfill cover soil, was selected as the attachment surface since it permits change of surface charge without affecting its optical properties. Glass surfaces can be

modified by chemical treatments where surfaces with the optical properties of glass but with different chemical properties are desired, e.g. neutral, hydrophobic or hydrophilic surfaces. The majority of microorganisms attach preferentially to hydrophobic surfaces although some bacteria (spore-forming bacteria) adhere preferentially to hydrophilic surfaces. Purging with the culture irrigation medium for five minutes to facilitate adsorption of solutes to the chamber surfaces prior to inoculation was undertaken to define the chemistry of the surface before adsorption or attachment of the microbial population.

Continuous Culture Microscopy Unit Calibration: Crucial and, potentially, rate-limiting events in biofilm formation are the transport of microorganisms to the solid-liquid interface and the subsequent attachment to the substratum. If the attached cells find suitable environmental conditions they should grow, replicate, and form a biofilm (Mueller, 1996).

The study of attached microorganisms under defined hydrodynamic conditions requires that the laminar flow velocity is controlled. This velocity is equal to the medium flow rate (ml s^{-1}) divided by the cross-sectional area (cm^2) of the chamber. During the supply of medium, small air bubbles may spontaneously form within the pump tubing. When these bubbles pass through the culture chamber, the movement of each meniscus along the glass surface may dislodge inadequately attached cells or alter the conformation of the microcolonies and their associated exopolymers. This is avoided by degassing (autoclaving) the influent medium.

Diffusion may play an important role in biofilms because under laminar flow conditions fluid near the edges moves more slowly than in the main steam and, hence, exchanges less rapidly. Bromophenol blue was chosen as an indicator as it has a molecular weight similar to hexanoic acid. The molecular weight of this dye is low so that irregularities in concentration smooth out rapidly. At the selected dilution rate the medium flowed through the CCMU at a rate which was sufficient for replacement to occur within 4s. The time concentration study showed that outflow of the dye was slower than its inflow. By looking at a single IA frame a gradient was seen between the wall of the CCMU and the centre of the chamber. In this study only the x and y planes were investigated (where the biofilm was situated). This coverslip boundary layer (z plane) could be investigated by focussing at different levels within the CCMU which would be investigated with 3D software (which is available and would be a good extension of this project). As all the growth measurements were made at the centre of the CCMU such gradients should have had little influence on the results obtained.

Dilution Rate: Detailed analysis of each component type of microorganism was not made as a presence or absence of growth of the associations was the analytical criterion. Optical density measurements, area colonized and number of organisms provided this information. In most of the individual studies, the measured optical density rapidly increased between the first and second determinations (24h) probably due to the supply of influent nutrient medium. Of the five dilution rates examined, enhanced (determined from colonized area, number of cells and optical density) biofilm development occurred at a dilution rate of $0.5h^{-1}$.

Substrate Concentration: Kim and Frank (1995) quantified, with computerized image analysis, biofilms cultured in the presence of amino acids by measuring the percentage of area colonized by cells. The degree to which the biofilms initially developed was associated with the amino acid concentration within the range 0.12 - $6g.l^{-1}$. A similar situation was found in this study where an increase in hexanoate concentration from 0 - 50mM resulted in a corresponding increase in all measured parameters.

In the present study, a control was necessary to ensure that oxygen (or some essential requirement for growth) did not become rate limiting within the microenvironment while the hexanoic acid concentration was growth limiting in the macro-environment. It was seen that as the concentration of hexanoic acid increased so did the biofilm biomass (during the period of analysis). Increases in substrate concentration in the CCMUs facilitated increased growth within the surface micro-environment (within the analysis period).

Detailed Investigation of Component Species: To ascertain if the applied dilution rate ($D = 0.5h^{-1}$) and highest substrate concentration (50mM) in the CCMUs permitted unrestricted microbial growth a detailed analysis of morphological categories was undertaken.

Microbial Colonization of a CCMU in the presence of 10mM Hexanoic Acid at a Dilution Rate of 0.5h^{-1}: By studying the different morphotypes among biofilm component species, both the associations and specific species can be monitored by image analysis during primary growth. The development of the image analysis programme allowed the distinguishing of various cell types. Posch *et al.* (1997) likewise distinguished a number (five) of major bacterial phenotypes based on visual observations.

The reason for a discriminated phase of > 100% when monitoring the microbial colonization of the CCMU was the measuring frame (Identframe) selected (3.3.5.xii). The number of fungal hyphae increased markedly between 108h and 132h cultivation. Bacterial species have higher growth rates so would initially outcompete the fungi for the nutrient(s) available. The delayed increase in fungal biomass may also have been due to previous growth of other species which then allowed the fungal hyphae to grow on the putative metabolic products. It is possible that due to the considerable intertwining of the hyphae the average elongation was underestimated. Image analysis is biased towards primary hyphae but any crossing fungal hyphae would be cut, hence reducing the average length. This possibility was supported by the observed increased surface area of fungal hyphae compared to fungal elongation. A more complicated, specifically fungus orientated, image analysis programme could solve this problem (Bolton *et al.*, 1991; Treskatis *et al.*, 1997).

Improvements in methodologies used to estimate number of microorganisms have resulted in the ability to generate data far more efficiently, and with greater accuracy, than previously possible (Treskatis *et al.*, 1997). The use of microscopy/image analysis to determine kinetic parameters of growth and to describe the time course of events such as surface colonization and biofilm development in a population of microorganisms was verified by the programmes developed during the course of this study.

CHAPTER SIX

6. SPECIFIC GROWTH RATE DETERMINATIONS OF MICROBIAL ASSOCIATIONS ISOLATED FROM A MUNICIPAL SOLID WASTE LANDFILL

6.1 *INTRODUCTION*

Although microbial surface-attached growth is important, many assumptions concerning microbial activity in the environment are based solely on studies of planktonic unicells (Brock, 1971; Fletcher, 1979; Shoeb, Tawfik and Shibl, 1991; Thomas and Paul, 1996). The attachment of bacteria to surfaces often reduces predation (Wilson, Enfield, Dunlop and Cosby, 1981), permits use of adsorbed substrate (Van Loosdrecht *et al.*, 1990), and positions cells in favourable environments (Wolfaard *et al.*, 1994). Attachment to plant or soil surfaces can result in a shift of diffusion gradients due to the formation of microbial biofilms (Stotzky, 1980). This increases the concentrations of nutrients within the microenvironment of the attached bacteria. Studies of these phenomena in continuous or batch culture are usually difficult or inappropriate as attached and planktonic cells respond differently to environmental perturbations and, thus, direct studies of surface growth are needed (Senior, 1991). The morphology of filamentous microorganisms is of great interest as it could be a visible expression of physiology and metabolism of the microorganisms (Treskatis *et al.*, 1997).

6.1.1 **Colonization Kinetics**

Colonization kinetics are necessary to calculate cell growth and attachment rates from cell density and distribution figures (Caldwell, Malone and Kieft, 1983; Escher and Charachlis, 1988). Attempts to quantify microbial growth rates on surfaces have been discussed by Brock (1971) and Busscher and Van Der Mei (1995). In early studies of microbial surface colonization Bott and Brock (1970a; b) used the exponential growth equation to determine the specific growth rate (μ) of bacteria. Subsequently, Caldwell *et al.* (1981) found that by using the exponential growth equation, the progeny of cells which attach during the cultivation period erroneously inflate the growth rate. These authors therefore derived a colonization equation which accounted for simultaneous attachment and growth during surface colonization.

The rate of cellular colonization of a substratum is the net result of attachment (e.g., adsorption, desorption) and growth processes (Fletcher *et al.*, 1980). During colonization of surfaces, bacterial cells frequently become arranged in a characteristic pattern that reflects the growth and attachment rate of the colonizing population. If it is assumed that the growth rate of cells within a microcolony is constant, the attachment rate is constant and no emigration occurs, it is possible to derive a surface colonization equation (Equation 6.1) which integrates attachment and growth to give the number of cells present as a function of the specific growth rate, attachment rate and the time of surface exposure (Caldwell *et al.*, 1981).

$$N\ (cells.\ field^{-1}) = \left(\frac{A}{\mu}\right) e^{\mu t} - \left(\frac{A}{\mu}\right) \qquad (6.1)$$

Where N = number of cells on surface (cell field^{-1});
A = attachment rate (cells field^{-1}h^{-1});
μ = specific growth rate (h^{-1}); and
t = incubation period (h).

The number of colonies containing four cells (two generations) theoretically becomes equal to the attachment rate (A) divided by the specific growth rate (μ) (Caldwell *et al.*, 1983). Equation 6.1 permits derivation of the surface growth rate equation by substituting colony number for A/μ in the surface colonization equation and gives the specific growth rate from the number of cells present, the number of colonies containing four cells and the time of surface exposure. Thus, it is possible to obtain the attachment rate and specific growth rate from the number and distribution of cells present during surface colonization (Caldwell, 1984).

In situations where cells are present in the influent medium and are continuously attaching, applying the colonization equation to describe simultaneous growth and attachment (Caldwell *et al.*, 1981) assumes the cells attach at regular time intervals and, subsequently, grow exponentially. This avoids an overestimation of the growth rate due to late attachment. The colonization equation diverges from the exponential growth

equation when the growth rate is low and the attachment rate is high. When the colonization equation approaches exponential at low attachment rates and high growth rates, the appropriateness of fit for field data would be comparable for both equations (Caldwell *et al.*, 1983).

6.1.2 **Objectives**

The objective of this component of the research programme was to determine if image analysis could be used effectively to calculate the specific growth rates of various undefined landfill microorganisms of an association enriched/isolated from municipal refuse (Inoculum three). The approach used was to attempt to determine (by different methods) the specific growth rates of the surface-attached microorganisms by monitoring both visually identifiable microbial cell types and total microorganisms at different times during biofilm growth. Increases in the numbers of the visually identifiable morphotyes over time were then compared to the specific growth rate of the same association grown as a planktonic culture.

6.2 *MATERIALS AND METHODS*

6.2.1 **Planktonic Species**

Preparation: Conical flasks (250ml) were plugged with non-absorbent cotton wool, covered with aluminium foil and autoclaved (121°C for 15 minutes). Sterile mineral salts solution (90ml) (see section 4.2.2), supplemented with 5, 10, 25 or 50mM hexanoic acid, was aseptically added to duplicate flasks which were then transferred to a Controlled Environment Incubator Shaker (New Brunswick Scientific Co. Inc, Edison, N.J.) and agitated at 150rpm and 30°C for 24h to acclimatized the media prior to inoculation.

Inoculation: To inoculate the flasks, inoculum (10ml) was taken from the aerobic CCU's (see section 4.2.6).

Growth Determination: Growth was determined by measuring optical density at a wavelength of 625nm (Milton Roy Spectronic 301 spectrophotometer) at specific time intervals. The specific growth rates of the microbial association were determined from the log phase of growth using Equation 6.2.

$$Specific\ Growth\ Rate = \frac{\log_e X_t - \log_e X_O}{t_t - t_0}\ (h^{-1}) \quad (6.2)$$

Where X_t = optical density at time t;

X_0 = optical density at time 0;

t_t = time of final optical density reading; and

t_0 = time of initial optical density reading.

Once the specific growth rates had been determined for the association at each hexanoate concentration they were plotted against the discrete concentrations of hexanoic acid to produce a Monod plot (Gottschal and Dijhkuizen, 1992).

6.2.2 Surface-Attached Species

Continuous Culture Microscopy Unit Preparation: The CCMUs were prepared as before (see section 4.2.7).

Irrigation Solution: The chemical components of the irrigation solution were as before (see section 4.2.2).

Influent Medium Substrate Concentration: The hexanoic acid concentrations used were 5, 10, 25 and 50mM. The dilution rate (D) was $0.5h^{-1}$.

Inoculation: The CCMUs were inoculated (0.085ml) from the aerobic CCU's. Twenty four h after inoculation the peristaltic pump (Ismatec IPN 24B) was started. The CCMUs were analyzed for growth 36, 48, 60, 72 and 94h after inoculation.

Growth Determination: Growth determination was made by image analysis as detailed in 4.2.7. Colonization by the microbial association was determined by image analysis at 400X magnification (equivalent to 4920μm^2) by different methods, viz: number of cells.field^{-1}; area colonized.field^{-1}; optical density.field^{-1}; and discriminated phase.field^{-1}. The log phase of growth was determined from the increases in area colonized by the microbial associations at the prescribed D value.

Specific Growth Rate of Microbial Association: The specific growth rates of the microbial associations were determined from the log phases of growth (determined from increases in number of cells, area colonized, optical density.field^{-1} and discriminated phase.field^{-1}) using Equation 6.2.

Specific Growth Rate Determinations of Microbial Inoculum three Components: Detailed specific growth rate determinations of morphologically identifiable association members were made at 1000X magnification, equivalent to 45.4834μm^2. The total (SUM) area (μm^2) colonized and number of the four distinguishable microbial cell types were determined by image analysis (Dudley, Senior, Bruton and Wallis, 1994c). The relative area of discriminated phase, area colonized (SUM) and number of microorganisms determined from distinguishable microbial cells.field^{-1} were used to determine the specific growth rate of Inoculum three.

6.3 *EXPERIMENTAL RESULTS*

6.3.1 **Planktonic**

Growth Curves with Increasing Hexanoic Acid Concentration: Planktonic associations were cultured in the presence of different concentrations of hexanoic acid (see section 6.2.1). As the hexanoic acid concentration was increased, the lag phase increased and the exponential growth phase continued for longer.

Specific Growth Rate: Figure 6.1 shows the specific growth rates of each of the planktonic populations of the three landfill associations.

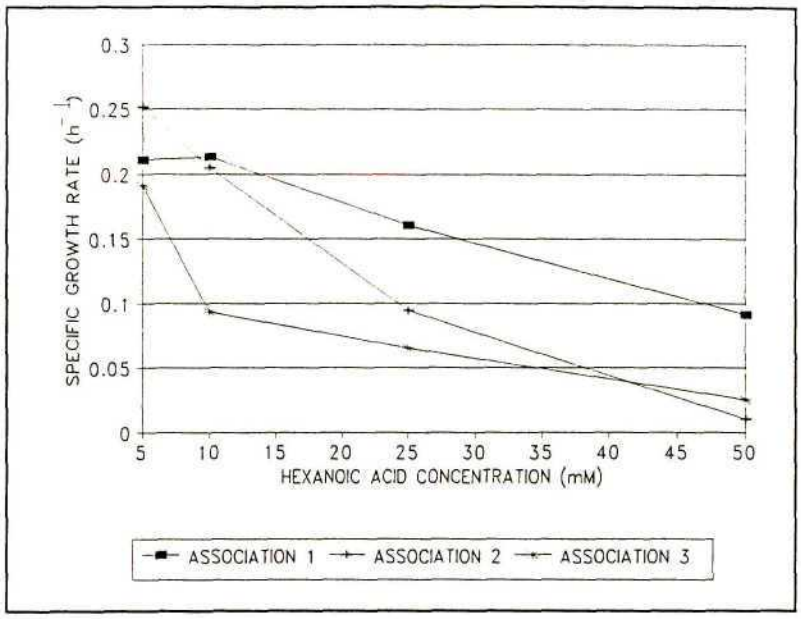
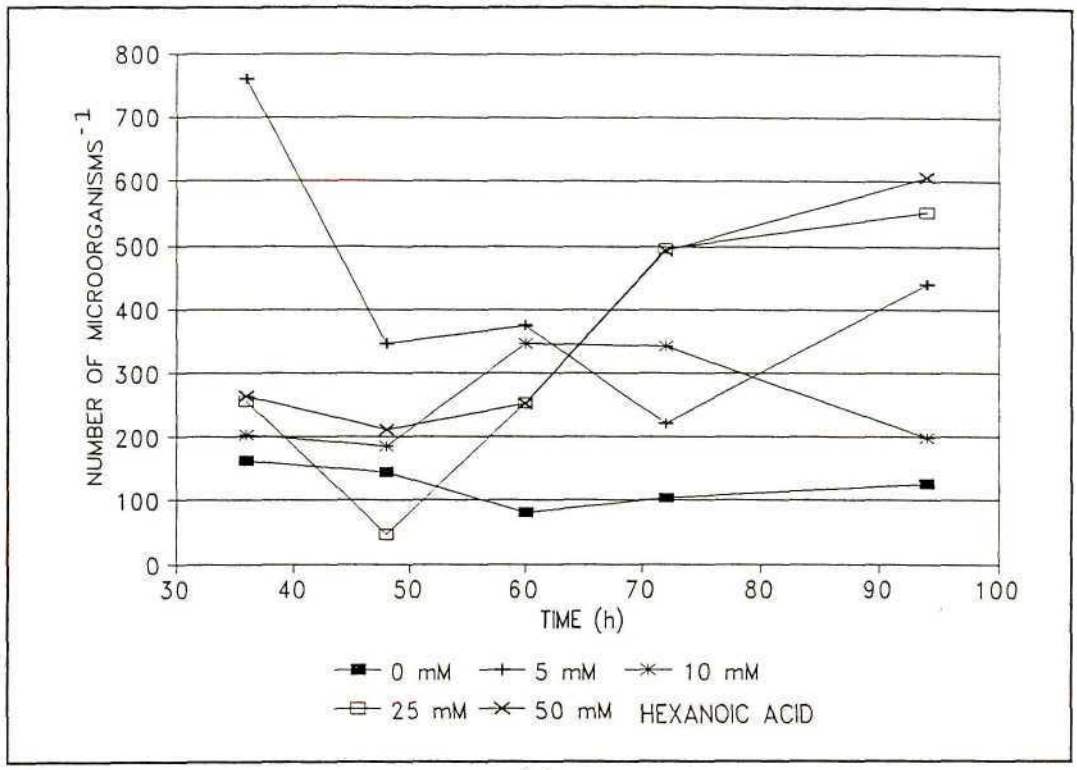


Figure 6.1. Specific growth rates of aerobic planktonic landfill associations in the presence of different hexanoic acid concentrations. The specific growth rates were determined from the log phases of batch cultures. Three enriched/isolated associations from a landfill were used: One. Soil cover; two. Soil from the landfill base liner where gas and leachate were actively emitted; and three. Municipal refuse.

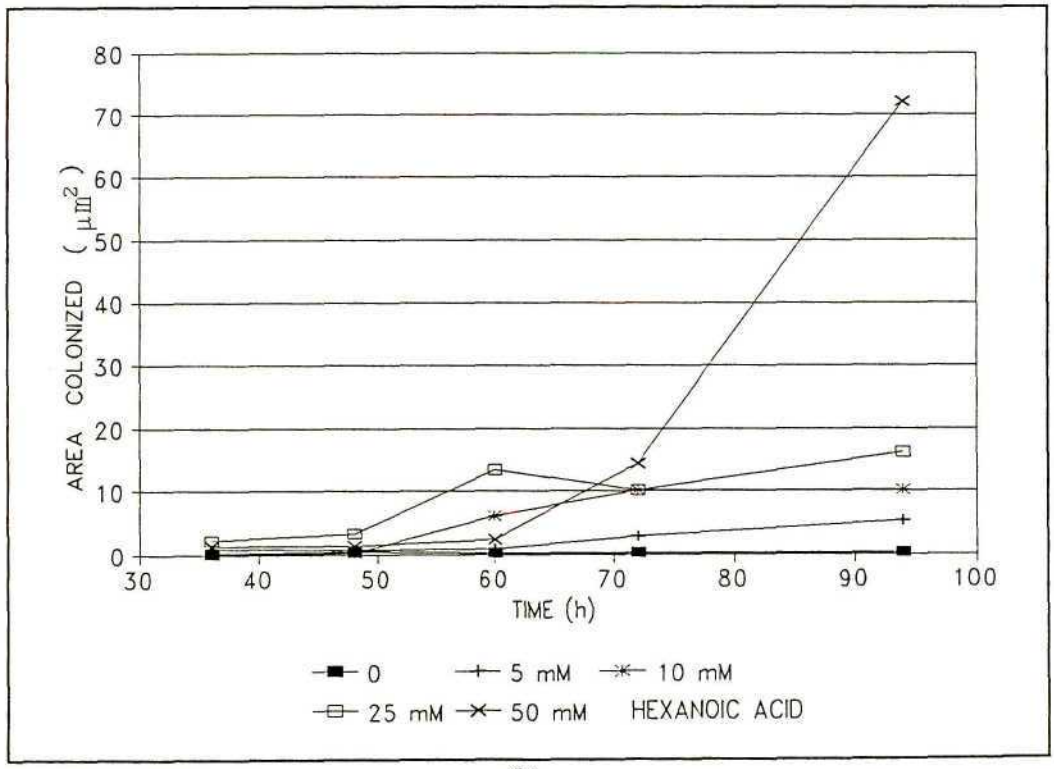
6.3.2 Surface-Attached Microbial Association

i. Growth Curves in the Presence of Increased Concentrations of Hexanoic Acid

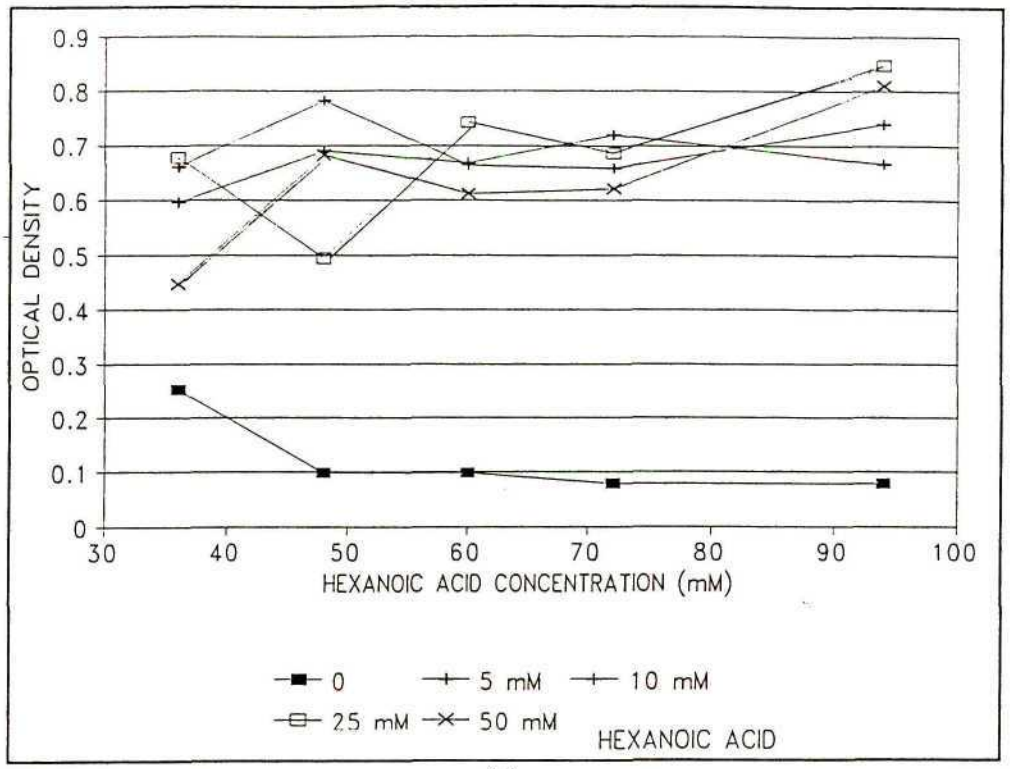
Figure 6.2 shows the growth curves of Inoculum three, determined by: (a) number of cells.field^{-1}; (b) area colonized.field^{-1}; and (c) optical density, in the presence of different hexanoic acid concentrations. The number of microorganisms appeared to fluctuate widely in the presence of the different hexanoate concentrations. This may have been due to some cells being initially washed out, reducing the number counted, followed by growth and increased cell number.


NUMBER OF MICROORGANISMS^{-1}
800
700
600
500
400
300
200
100
0
30
40
50
60
70
80
90
100
TIME (h)
0 mM
5 mM
10 mM
25 mM
50 mM
HEXANOIC ACID

(a)


AREA COLONIZED (μm^2)
80
70
60
50
40
30
20
10
0
30
40
50
60
70
80
90
100
TIME (h)
0
5 mM
10 mM
25 mM
50 mM
HEXANOIC ACID

(b)



(c)

Figure 6.2. Growth curves ((a) Number of microorganisms; (b) Area colonized; (c) Optical density) of the surface-attached members of Inoculum three, in the presence of 0, 5, 10, 25 and 50mM hexanoic acid. (Error bars are absent due to statistical limitation with only a single replication).

The control experiment showed an initial reduction in number of cells (from 162 cells.field^{-1} to 81 cells.field^{-1}) followed by a slight increase (126 cells.field^{-1}) (Figure 6.2a). With 5mM hexanoic acid the highest number of microorganisms (760.field^{-1}) was recorded after 36h. The number of cells then decreased until 72h (220.field^{-1}) and finally increased at the end of the experiment (441.field^{-1}). This suggested that 5mM hexanoic acid was inimical to surface-attached species. In contrast, the results with the higher hexanoic acid concentrations (25 and 50mM) showed substantial increases in cell number with time (553 cells.field^{-1} at 94h in 25mM hexanoic acid concentration).

Specifically, with 50mM hexanoic acid the colonized area increased dramatically (to a maximum > 72μm^2 at 94h). Growth, however, was specific to certain microbial components rather than general to the whole association over the time studied. The

association components responded equally to the increased concentration of hexanoic acid once the fungal hyphae played a dominant role.

ii. Specific Growth Rate

The specific growth rates of the surface-attached associations were determined (t = 94h) by three means: area (μm^2) colonized; total number of microorganisms.field^{-1}; and optical density (Figure 6.3). 10mM hexanoic acid yielded the highest specific growth rate determined from the measured increases in the area colonized (> 0.16 h^{-1}) and in the number of microorganism.field^{-1} (> 0.05 h^{-1}) with time (t = 94h). In contrast, optical density determinations showed the highest specific growth rate (0.01 h^{-1}) to occur at 25mM hexanoic acid.

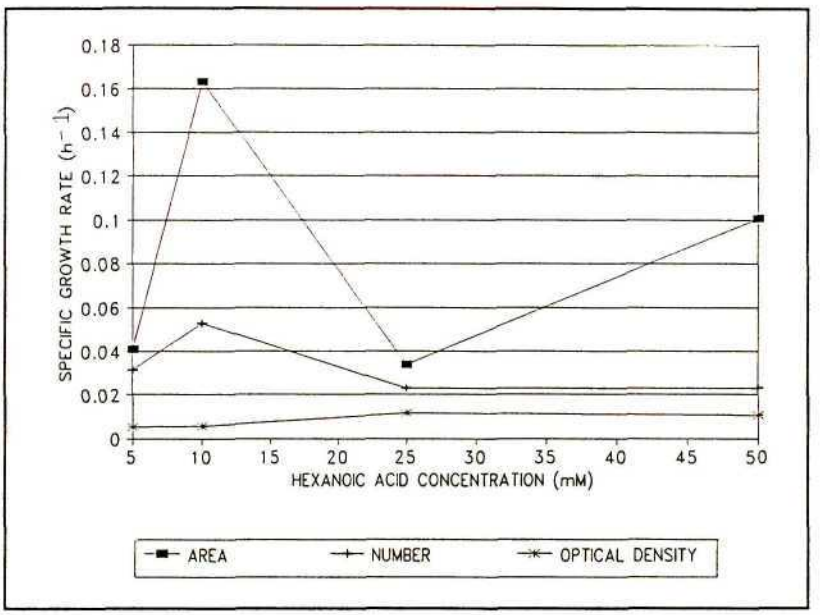


Figure 6.3. Specific growth rates determined by changes in colonized area (μm^2), number of organisms and optical density at 94h of the surface-attached members of Inoculum three in the presence of 5, 10, 25 and 50mM hexanoic acid.

6.3.3 Determinations of Area Colonized by and Number of Cells of Surface-Attached Microbial Association Component Species

i. Growth Curves Plotted from Area Colonized and Number of Microorganisms

The growth curves of the microbial components determined by colonized area (SUM) and number of microorganisms are shown in Figure 6.4.

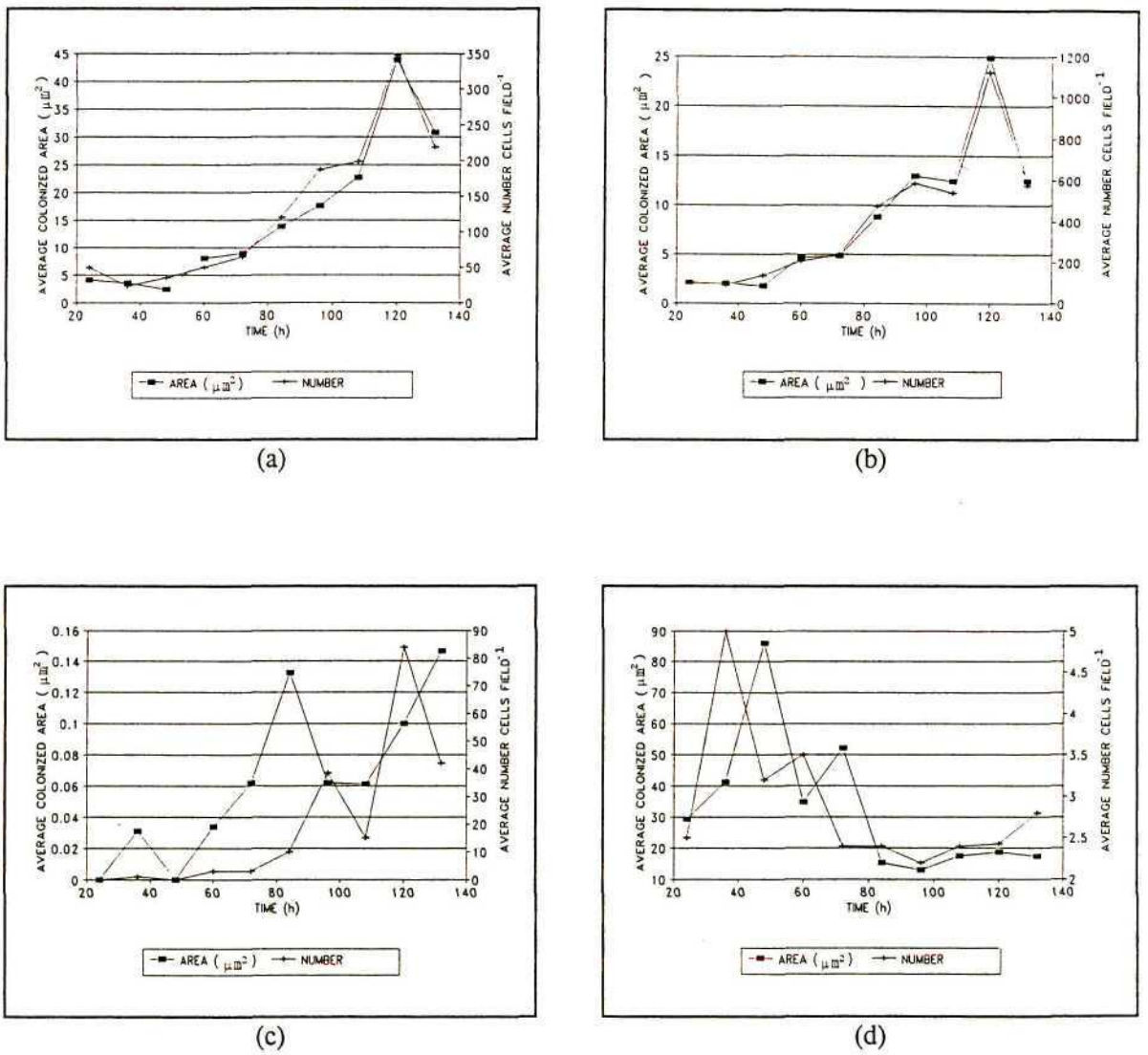


Figure 6.4. **Growth curves, expressed as area colonized and cell numbers, of: (a) Rods; (b) Cocci; (c) Fungal hyphae; and (d) Fungal spores of Inoculum three in the presence of 10mM hexanoic acid.**

The number of rods and area colonized increased over the period 48h to 120h (35 rods.field^{-1} and 2.34μm^2 at 48h to 347.field^{-1} and 43.8μm^2 at 120h) (Figure 6.4a). The cocci showed similar results (134 cocci.field^{-1} and 1.72μm^2 at 48h to 1123.field^{-1} and 24.8μm^2 at 120h) (Figure 6.4b). Fungal growth remained virtually static for two days following inoculation and then commenced, attaining a value of 0.03μm^2.field^{-1} at 60h. Rapid growth ensured over the next 24h period (0.13μm^2.field^{-1} at 84h) followed by a decline (0.06μm^2.field^{-1} at 96h) and then a second period of rapid growth (0.15μm^2.field^{-1} at 132h) (Figure 6.4c). The fungal spores decreased in both area (86μm^2.field^{-1} at 48h and 18.6μm^2.field^{-1} at 120h) and number (3.2.field^{-1} at 48h and 2.4.field^{-1} at 120h) with time (Figure 6.4d).

ii. Specific Growth Rate

The specific growth rate of each microbial component of Inoculum three was determined in the presence of 10mM hexanoic acid (Figure 6.5). With the exception of fungal hyphae, the number of cells and area colonized values were similar. This discrepancy was probably due to an increase in size but not in number of fungal hyphae, the branching effect mentioned before possibly contibuting as the fungal mycelium develops. In such a case the area calculation (> 0.11 h^{-1}) would be the most accuate measurement to use.

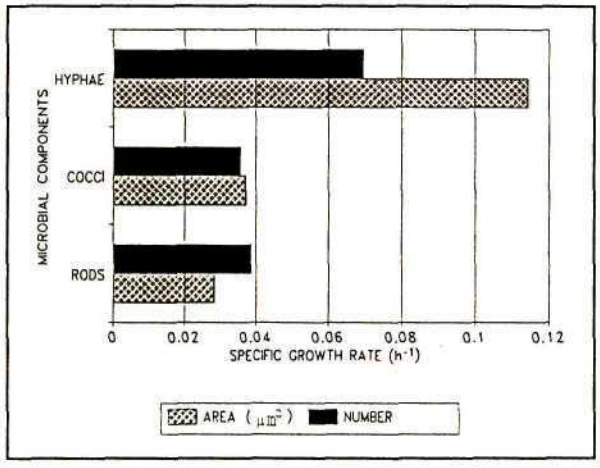


Figure 6.5. Specific growth rates (determined by number of cells and area colonized (SUM)) of microbial components of Inoculum three in the presence of 10mM hexanoic acid.

6.3.4 Determinations from Discriminated Phase, Area Colonized and Number of Microorganisms Calculated for Component Species of a Surface-Attached Microbial Association

Figure 6.6 shows the growth curves for the total area (μm^2); relative area of discriminated phase (total of all classes of micoorganisms).field^{-1} and average microbial number.field^{-1} during cultivation in a CCMU in the presence of 10mM hexanoic acid. The numbers began to increase from 48h and reached a peak at 120h when over 1400 microorganisms.field^{-1} of view were present. Increases in number of microorganisms and relative area of discriminated phase were recorded between 24h and

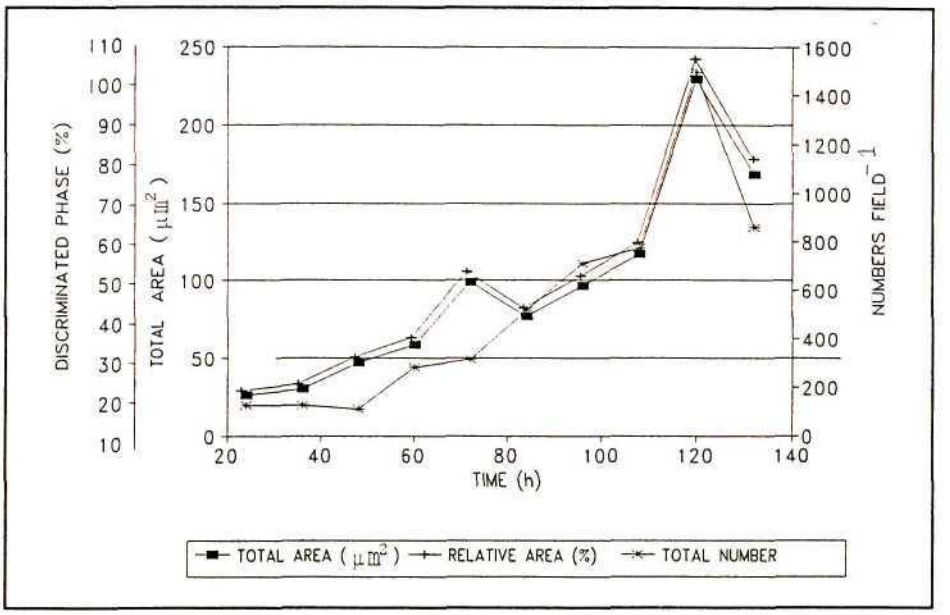


Figure 6.6. Growth curves derived from total area (μm^2); relative area of discriminated phase and microbial number.field^{-1} of surface-attached Inoculum three, in the presence of 10mM hexanoic acid.

The area colonized by the microorganisms only slowly increased. Relative area of discriminated phase, area colonized (SUM) and number of microorganisms.field^{-1} calculated for the component species were used to determine the specific growth rate of the association. Table 6.1 shows the respective specific growth rates determined from these parameters for Inoculum three. The rates determined from the relative area of

discriminated phase and the area colonized were similar. In contrast, the specific growth rate determined from the microbial number was higher.

Table 6.1. Specific growth rates (t = 132h) determined from area colonized, relative area of discriminated phase and microbial number of Inoculum three in the presence of 10 mM hexanoic acid (D = 0.5h^{-1}).

METHOD OF SPECIFIC GROWTH RATE DETERMINATION	SPECIFIC GROWTH RATE (h^{-1})
RELATIVE AREA OF DISCRIMINATED PHASE (%)	0.023
AREA (SUM) (μm^2)	0.023
MICROBIAL NUMBER	0.027

6.4 *DISCUSSION*

Image analysis was used to calculate the specific growth rates of various unidentified landfill component microbial species and associations by four different methods (area colonized, number of cells, optical density and discriminated phase). In measuring object specific (individual) counts (1000X magnification) it is simple with image analysis to obtain field-based parameters. The component species determinations were used to obtain the field-based parameters (area colonized, number of cells and discriminated phase) to determine the specific growth rate of the association. This was compared to the specific growth rate previously calculated for the mixed population at the lower magnification (400X).

To calculate the growth rate, the incubation period(s) must be known. In addition, the number of cells must be determined at discrete times. For the calculations, it is assumed that the cells included in the determinations belong to one component population based on morphological identification (Bott and Brock, 1970a; Westlake *et al.*, 1995). An "apparent μ" for the entire microbial community is of value in studies of net microbial activity. Particular cells in a population may attach but neverless not form a colony.

Therefore, only colonies with two or more cells (assumed to be actively growing) should be included in the determinations. Unfortunately, this procedure excludes those cells which have attached but not had sufficient time to divide. This is corrected by counting all the cells in the field by returning repeatedly to the same field of view after allowing sufficient time between viewing for the cells to grow/divide.

The colonization equation most accurately describes microbial growth on surfaces where the cells/microcolonies remain attached after the initial attachment process. The equation is not required where there are no cells in the influent stream (medium) since under such circumstances there is no further attachment after the initial attachment. In such cases the exponential equation can be used. When using the exponential growth equation to describe microbial surface colonization, cells are assumed to attach only at time zero and then grow exponentially. It is not assumed that there is no attachment during the incubation period as progeny of attached cells will also attach. Cells which are not attached will be washed out of the system and, hence, do not need to be included in the equation (at the dilution rate used in the experiment). The design of the CCMUs was such that all cells (attached/unattached) were counted.

Many theories of microbial growth and activity in the environment are based solely on batch or continuous mono-planktonic culture experiments. This is due, in part, to the lack of adequate kinetic data describing microbial growth on surfaces. Thus, these theories have not contributed directly to our knowledge of microbial activities in most environments and only identify a range of possibilities. Monitoring the initial stages of cell attachment and subsequent growth of a biofilm by image analysis facilitates more careful analysis of *in situ* microbial surface colonization (Moller *et al.*, 1995). The effects of selected components of landfill leachate components on microbial attachment and growth may then be quantified.

Changes in bacterial number on surfaces are due to numerous factors including: attachment (immigration); emigration; predation; growth; death; interactions between colonies; and modifications within colonies. The analysis of surface colonization can, however, be simplified by examining initial growth phases on newly-exposed surfaces. In this situation, most factors are relatively unimportant compared to the combined effects of attachment and growth (Caldwell *et al.*, 1981).

Planktonic: The specific growth rate of planktonic cells was higher than that of surface-attached cells in the presence of 50mM hexanoic acid. With lower hexanoate concentrations, the planktonic association exhibited a higher specific growth rate than similar associations growth as a biofilm (as would be expected when the association grows at K_{max}). The planktonic studies determined that 10mM hexanoic acid yielded the maximum growth while with the surface-attached studies 50mM heaxanoic acid was the optimum growth prerequisite. The three isolated associations exhibited similar results at the four hexanoic acid concentrations studied.

Surface-Attached Microbial Associations - Growth Curves in the Presence of Increased Concentrations of Hexanoic Acid: In this study, growth of the selected association was exponential but unbalanced (i.e., not all monitored parameters increased at the same rate) and, thus, was in agreement with the results of Caldwell *et al.*, (1981). In contrast to the earlier analysis, the growth curves determined from optical density showed inconsequential increase until 94h. Optical density would be the parameter to monitor once the biofilm was thick, making individual cell counts difficult. In the control there was a marked reduction in optical density compared to the other measurement parameters.

Each parameter selected had advantages and disadvantages in monitoring the growth of the biofilm. Image analysis allows combination of a number of different parameters to monitor biofilm growth.

Specific Growth Rate: Colonized area determination gave the highest specific growth rates under the growth conditions imposed. This was not necessarily accurate but of the three parameters used, area colonized furnished the highest increases during the study. The number of microorganisms enumerated at the highest magnification may not be a good value to use but optical density showed a small increase and as such could be a useful tool at high magnification.

Colonized area provided a criterion to quantify changes in the growth rate within a single division cycle. However, this method of analysis is only appropriate for cells which attach horizontally to the surface and form microcolonies of horizontal monolayers of cells.

Integrated optical density may be necessary to quantify the growth of organisms with other colonization patterns.

Caldwell *et al.* (1981) found that the use of cell number as a measure of biomass was inadequate for studying the initial phase of surface colonization. This was due to the periodicity of the cell division cycle. This was not confirmed in the present study due to disparity in the number of cells analyzed (many thousands) compared with the < 64 cells studied by Caldwell *et al.* (1981).

Microbial Association Determinations from Discriminated Phase, Area Colonized and Number of Microorganisms Calculated from Component Species: Monitoring growth of the component species allows a more accurate determination of the specific growth rate but, due to biofilm growth, this is of limited duration (< 120h in this study). The specific growth rates determined for the component species were higher than that determined for the whole association.

The various ways of determining the specific growth rate gave different results. Any one of these may be the "correct" answer under the culture conditions imposed. Depending on the state (thickness) of the association (free-living, mono-layer or thick biofilm) different monitoring methods may be employed to determine growth. Optical density would be useful when a biofilm is thick and the component populations are difficult to distinguish. Area colonized was a reliable parameter to monitor colonization while cell number was reliable while the component populations could be differentiated.

As a consequence of the results of this study, the kinetics of microbial colonization of surfaces *in situ* may be subjected to the same degree of mathematical analysis as the kinetics of homogeneous cultures. This type of analysis is needed if quantitative studies of microbial growth are to be extended to surfaces in various natural and artificial environments.

7. CONCLUDING REMARKS

This study was aimed primarily at method development and there are a number of areas/fields were this technique could be applied. Image analysis has now been developed to the extent where it can be used routinely in the laboratory for conventional analyses, including plate counts, contamination in large samples and analysis of CCMU microbial populations. The greatest advantage of image analysis in the study of microbial ecology is that the examination can be undertaken without disturbing the habitat, along with the large numbers of cells which can be observed and measured. The interactions between morphotypes (due to the recognition features of the developed macros) comprising the biofilm can be monitored in greater detail during growth by image analysis. Experimental programmes need to be designed with the above in mind. If possible the use of confocal laser scanning microscopy (CLSM) should be used in long term biofilm growth studies (Massol-Deyà *et al.*, 1995) but lack of a CLSM need not restrict the way forward.

In biofilm colonization studies, it is beneficial to retrieve images of the objects outside the depth of focus of the microscope objective. Lawrence *et al.* (1989) described a method of combining images to increase the "effective" depth of field at high magnification. Many smaller cells were detectable in the resultant high contrast combined images and should be explored further with a high powered computer.

The average rod surface area fluctuated while the average elongation (length) of the rods remained constant throughout the duration of biofilm development. There was no discrimination of cells at different stages in the cell cycle in this study which may prove illuminating.

There is limited literature describing the quantification of mixed culture acclimatization especially in a kinetic context. Species interactions and co-metabolism, which can render a refractory compound biodegradable, are extremely important phenomena. These should be further investigated by image analysis.

It is possible that due to the considerable intertwining of the fungal hyphae the average elongation was underestimated. The current Image analysis programme is biased towards primary hyphae so that any crossing fungal hyphae would be cut, hence reducing the

average length. A specifically fungus orientated programme could solve this problem (using skeletonized functions) which needs greater computer power than that of the Kontron system.

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Table A.1.1 shows the gas chromatography peak retention times for hexanoic acid catabolic intermediates. A typical standard from which residual hexanoic acid concentrations were estimated by GC analysis is shown in Figure A.1.1. The graph is linear over the hexanoic acid concentration range of 0 - 50mM.

Table A.1.1. **Gas chromatography peak retention times for standards of hexanoic acid and putative breakdown products. The GC operating conditions are detailed in 2.1.1.**

PEAK	RETENTION TIME (MIN)
PRESSURE PEAK	0.7
PROPIONIC ACID	1.8
ACETIC ACID	2.4
BUTYRIC ACID	3.3
HEXANOIC ACID	6.8

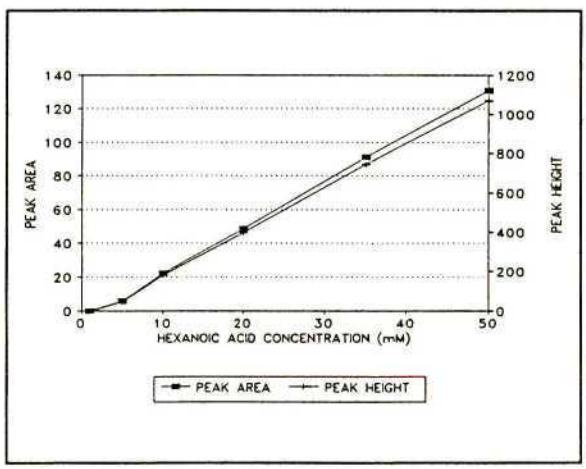

Figure A.1.1. **Typical GC standard from which residual hexanoic acid concentrations were estimated. Hexanoic acid concentrations were determined by peak area comparison with the standards.**

Analogue to digital converter - a device which senses an analogue signal and converts it into a proportional representation in digital form.

Bernoulli disk - removable cartridgesof 44 megabyte capacity for image storage.

Binary image - an image where pixel intensities take only two possible values, either zero or a specific non-zero value (typically unity or full-scale). Binary images take much less space to store in the image memory of the computer or on a disc and they are very convenient for making measurements, eg. area or perimeter of a feature. Often a binary image may be shown as white (for non-zero) and black (for 0).

Binary image operations - calculations on a binary image. These include:

Cutlink - manually separates adjoining objects.

Erosion - the removal of a layer of pixels around a feature. Small features may be totally removed and/or separated into their components.

Dilation - the reverse of erosion; adding a layer of pixels around a feature. Thus, features which are fragmented may be joined.

Scrap - allows objects smaller than a particular threshold size, eg. 20 pixels, to be removed from the image.

CCD camera - a video camera containing a Charge-Coupled Device which is an integrated circuit in the form of a semiconductor light sensor.

Contour overlay - Copies the contour lines of binary objects of image input one, together with the contents of image input two, into the overlay or a selected grey level of image output.

Contrast - a measure of the variation in brightness between the lightest and darkest portions of a given image.

Contrast normalization - mainly used in automatic measurement sequences to enable segmentation from fixed threshold levels. The darkest and the brightest pixels in an image are calculated. The darkest level is transformed to grey value 0, the brightest pixel is transformed to grey level 255. The resulting image contains the full intensity dynamic range.

Densitometric parameters - based on the evaluation of grey-levels.

Digitization - conversion of analogue information to digital form.

Digitizing tablet - a tablet on which the features of interest in a specimen can be drawn with a hand-held cursor or electronic "pen". Output is shown on the screen and can be either added to images or measured.

Enhancement - a range of techniques used to modify the appearance of an image to make it visually more attractive or to improve the visibility of certain features. The aim is to make the analysis of the image easier for a human observer or for subsequent machine-based processing.

Feature - part of an image which can be isolated from the remainder by some means. For example, a "particle" can often be distinguished by having brighter intensity than its surroundings. Separation of an image into features is referred to as "segmentation".

Feret diameter - The longest diameter of an object obtained by selecting the largest of the Feret diameters measured in 32 directions (i.e at an angular resolution of 5.7 degrees).

Field-measurements - measure field-based parameters from a binary image and store them in a data base. One feature vector per image is produced.

Filter - a device, method or programme alogarithm which separates signals or other data based upon specified criteria.

> **Lowpass filter** - mainly used for smoothing and homogenizing textured structures.
>
> **Sigma filter** - because background electronic noise is a statistical event, this filter calculates the standard deviation in a matrix and smooths pixels which deviate from the mean grey-level.

Frame grabber - an input device which captures an image from a video camera, digitizes the analogue signal into a defined number of bits per pixel and transmits it to some form of digital memory.

Grey-level intensity - the brightness of each pixel in the image.

Grey-level image - often known as a grey scale image, or grey value image: an image in which the regions or points may take on a range of values or grey-levels (distinguished from a *binary* image in which only two are allowed). To match a conventional 635 line TV camera, a typical grey image consists of 512 lines with 512 pixels on each line, i.e. 262,144 pixels.

Grey-level histogram - in digital image processing, "histogram" refers to the distribution showing the number of pixels which have a particular intensity level.

Image - a two-dimensional representation of the response from some sensing device responding typically, but not necessarily, to light or other radiation. The process of digitization produces a *digital image* consisting of a table of numeric values which describe the relative positions and values of the original responses.

Image analysis - an operation or set of operations designed to give a numerical or logical result from selected image features which can be expressed in non-image terms (such as area, shape, size distribution). Image analysis is often preceded by image processing.

Image processing - refers to digital or analog enhancement and geometric manipulation of the video signal. Image processing is the set of methods used to enhance the image prior to image analysis.

Intensity - a term which is used rather loosely in this context, indicating the number for a given pixel in the digitized image.

Look-up table (LUT) - a means whereby the digitized video signal may be altered to produce a more readily interpreted display. Instead of displaying an image so that the brightness of each pixel is proportional to its numerical value, the values in the stored digital image can be converted on display to a different value using the look-up table. A grey image can, therefore, be displayed as a negative, or as a part of the range of grey values expanded, without changing the stored data. By giving different tables for different colours the original grey image can be displayed in false colour. A look-up table can also be applied to the input from the TV camera.

Macros - comprise a series of rudimentary computer commands which are used to execute an image analysis measurement routine. Normally, macros are used to create executable procedures which can be defined, edited, stored, loaded and executed.

Neighbourhood (or local) operations - operations on an image using the neighbourhood of each pixel to calculate a new value for that pixel. Typically a 3X3 or 5X5 group of pixels is used to "smooth" an image or enhance some aspect of the image, such as the edge of a feature.

Object - in a binary image, a region in which all the pixels are connected (according to the connectivity rules that apply in the image analysis system), and which is surrounded by pixels of the another type.

Object-measurements - measurements which relate to the individual objects in the image, and which can be combined with others on an object-by-object basis to give other information - for example, elongation of roundness (FCIRCLE).

Overlay - superimposition of a binary image, graphics or text over a grey image, using colour or good contrast to differentiate between the two images.

Pixel (or picture element) - normally refers to one of the grid points on the 2-D grid or the corresponding element in the 2-D array of intensity values representing the digitized image.

Point spread - the continuous function which describes the result of applying a point source to a linear system. A digital filter can be defined as a sampled version of this function. The result is a convolution mask.

Resolution - a measure of how accurately a sampled image represents the actual scene. Resolution can be used as a spatial measure or as an indicator of how faithfully brightness or colour are represented.

Segmentation - the process of separating an image into the various features and discriminating those parts of an image which are of interest from those which are not (the "background"). The simplest type of segmentation is thresholding.

Shape factor - a number derived from two or more measurements which indicates something particular about the shape of the feature. There are many different shape factors, eg. maximum diameter and minimum diameter will be large for needle-like features but near unity for circular objects. As an indicator the shape factor of a perfect circle is 1. Measurements are combined to form a factor which is dimensionless and, thus, independent of the size or positioning of the feature.

Thresholding - the simplest process for converting a grey-level image to a binary image by comparing each pixel value to a *threshold* value. As an example those pixels exceeding the threshold become white, while the remainder become black.

Visual textures - (mathematically not well defined) description of local grey-level changes in objects or regions.

APPENDIX THREE : MACROS USED TO QUANTIFY SURFACE-ATTACHED MICROBIAL GROWTH

```
### BACKGROUND CORRECTION ###

macro bgcor
clearmes
read "CREATE (1) a new BACKGROUND image OR use CURRENT one (2)
: ",answer
if answer eq 1
clearmes
read "What image DO YOU WANT TO USE : ",n
display n
read "What no. do you want the output image to be : ",p
read "What size [15] is the largest object in this images : ",m
read "What is the OFFSET (5)- diff between original + filter image : ",o
wrte "IT IS SET FOR BRIGHT OBJECTS ON A DARK BACKGROUND"
display n+1
trackgr n,n+1,m,o,_ON
clear p,0
display n+1
shaddef n+1,n+1
wrte "The shading correction is set to additive (ILLUMINATION)"
display p
shadcorr n,p,n+1,_ON,0
endif
 if answer eq 2
read "What image is to be CORRECTED : ",o
read "What image is the OUTPUT : ",p
read "What image is the SHADING REFERENCE IMAGE : ",l
wrte " THE SHADING CORRECTION IS FOR ILLUMINATION (additive) "
shadcorr o,p,l,_ON,0
 endif
endmacro


### IMAGE PROCESSING ###

macro bimp
clearmes
wrte "MACRO USED FOR BINARY IMAGE PROCESSING"
read "WHICH IMAGE DO YOU WISH TO USE..... ",n
display n
wrte "IMAGE No. ",n," WILL BE PROCESSED"
wrte "SELECT BINARY IMAGE PROCESSING REQUIRED"
wrte " 1 - GREY-LEVEL OR BINARY"
wrte " 2 - BINARY ONLY"
read " YOUR SELECTION IS : ",answer
clearmes
#IMAGE PROCESSING - GREY-LEVEL OR BINARY IMAGE
 if answer eq 1
wrte "SELECT THE BINARY IMAGE PROCESSING REQUIRED : "
wrte " 10 - ERODE 11 - DILATE"
wrte " 12 - OPEN 13 - CLOSE"
wrte " 14 - review sequence"
 read "YOUR SELECTION IS : ",answer
  if answer eq 10
read "WHAT SHAPE (1-8, [7]) TO USE : ",m
read "WHAT PHASE (grey-level) [255] TO USE : ",p
read "NUMBER OF COUNTS (erosions) [1] TO USE : ",o
erode n,n+1,m,p,o
 endif
 if answer eq 11
read "WHAT SHAPE (1-8, [7]) TO USE : ",m
read "WHAT PHASE (grey-level) [255] TO USE : ",p
read "NUMBER OF COUNTS (erosions) [1] TO USE : ",o
dilate n,n+1,m,p,o
 endif
 if answer eq 12
read "WHAT SHAPE (1-8, [7]) TO USE : ",m
read "WHAT PHASE (grey-level) [255] TO USE : ",p
read "NUMBER OF COUNTS (erosions) [1] TO USE : ",o
open n,n+1,m,p,o
 endif
 if answer eq 13
read "WHAT SHAPE (1-8, [7]) TO USE : ",m
read "WHAT PHASE (grey-level) [255] TO USE : ",p
read "NUMBER OF COUNTS (erosions) [1] TO USE : ",o
close n,n+1,m,p,o
endif
 if answer eq 14
loadlut "grey"
read "WHAT no. start image : ",m
read "WHAT no. of images in sequence : ",p
review m,p,0,1,_OFF
 endif
endif

#IMAGE PROCESSING - BINARY IMAGE
 if answer eq 2
wrte "SELECT THE BINARY IMAGE PROCESSING REQUIRED : "
wrte " 20 - MARK OBJECTS 21 - SCRAP "
wrte " 22 - THINNING 23 - FILL"
wrte " 24 - CONTOUR 25 - MASK"
wrte " 26 - LINK LINES 27 - REJECT OBJECT"
wrte " 28 - CUTLINK "
 read "YOUR SELECTION IS : ",answer
 if answer eq 20
read "IMAGE ONE : ",m
read "IMAGE TWO : ",p
read "RESULTANT IMAGE : ",o
markobj m,p,o,_ON,_ON,_OFF
 endif
 if answer eq 21
read "WHAT AREA FOR THE LOWER LIMIT [0]) : ",m
read "WHAT AREA FOR THE UPPER LIMIT [30] : ",p
scrap n,n+1,_OFF,m,p,_ON,_ON
stop
 endif
 if answer eq 22
read "HOW MAY STEPS [0] : ",m
read "WHAT MODE [0] : ",p
thinbin n,n+1,m,p,_OFF,_ON
 endif
 if answer eq 23
fill n,n+1
 endif
 if answer eq 24
read "IMAGE ONE : ",m
read "IMAGE TWO : ",n
read "RESULTANT IMAGE : ",o
read " WHAT SHAPE : ",p
contour m,n,o,p,128,_ON,_ON
stop
 endif
 if answer eq 25
read "IMAGE ONE : ",m
read "IMAGE TWO : ",n
read "RESULTANT IMAGE : ",o
read " WHAT SHAPE : ",p
mask m,n,o,255,p,_OFF
 endif
 if answer eq 26
read "HOW MAY DILATION STEPS [7] : ",m
read "HOW MANY THINNING STEPS (not 7) [0]: ",p
linklin n,n+1,m,p
 endif
 if answer eq 27
wrte "THIS MUST BE AN IDENTIFIED IMAGE....."
rejectobj n,n+1,0
 endif
endif
 if answer eq 28
cutlink n,n+1,8,1,0,_ON
 endif
endif
endmacro


### DATA BASE INFORMATION ###

macro dbinf
text1="co-2"
mogrtxt4="INFO"
read "which Data Base do you wish to add data to : ",text1
read "TEXT INFO to ADD TO DATA BASE : ",mogrtxt4
for i=1, i<2, i=i+1
   name1=text1
   DBopen name1
   DBsetinfo name1,mogrtxt4
   DBclose name1,name2
   wrte text1," DB added to > ",mogrtxt4
endfor
endmacro


### DELETION OF DATA BASES ###

macro deldb
clearmes
answer=0
read "DO YOU WISH TO DELETE THE DATA BASES (1) or NOT (2) :
",answer
if answer eq 2
stop
endif
if answer eq 1
wrte ".....deleting data bases"
DBerase "MOGR-DB1"
Dberase "MOGR-DB2"
Dberase "MOGR-DB3"
Dberase "MOGR-DB4"
endif
endmacro


### FILTERS ###

macro filtr
clearmes
wrte "MACRO USED FOR IMAGE ENHANCEMENT - FILTERS"
read "WHICH IMAGE DO YOU WISH TO USE..... ",n
wrte "YOU HAVE CHOSEN IMAGE No. ",n," TO BE ENHANCED"
display n
clearmes
clear n+1,0
wrte "SELECT THE TYPE OF FILTER REQUIRED "
wrte "1 - CONTRAST 2 - SMOOTHING"
wrte "3 - SHARPENING 4 - EDGES"
read " YOUR SELECTION IS : ",answer
 if answer eq 1
wrte "SELECT THE CONTRAST FILTER REQUIRED."
wrte " 10 - NORMALIZATION 11 - LINEARIZATION"
wrte " 12 - SCALED "
 read "YOUR SELECTION IS : ",answer
 if answer eq 10
display n+1
read "WHAT LEVEL OF NORM [2] DO YOU WISH TO USE.....",p
clear n+1,0
normim n,n+1,p
 endif
 if answer eq 11
display n+1
linhist n,n+1
 endif
 if answer eq 12
display n+1
wrte "THIS REQUIRES ONE TO INTERACTIVELY SET THE LEVELS"
scalint n,n+1,64,191,_ON
 endif
 endif
 if answer eq 2
clearmes
wrte "SELECT THE SMOOTHING FILTER REQUIRED :"
wrte " 15 - LOWPASS 16 - SIGMA"
wrte " 17 - MEDIAN 18 - RANK"
wrte " 19 - Non Linear Lowpass 20 - Weighted LowPass"
wrte " 21 - GAUSSIAN lowpass filter"
read "YOUR SELECTION IS : ",answer
 if answer eq 15
display n+1
read "WHAT SIZE MATRIX (X) DO YOU WISH TO USE.....",m
read "WHAT SIZE MATRIX (Y) DO YOU WISH TO USE.....",p
read "HOW MANY TIMES DO YOU WISH TO DO IT.....",o
lowpass n,n+1,m,p,o
 endif
 if answer eq 16
display n+1
read "WHAT SIZE AVERAGE MATRIX TO USE..... ",m
read "WHAT SIGMA (1-127, [15]) TO USE..... ",p
sigma n,n+1,m,p
 endif
 if answer eq 17
display n+1
read "WHAT SIZE MATRIX (odd; 3-15) TO USE..... ",m
median n,n+1,m
 endif
 if answer eq 18
display n+1
read "WHAT SIZE MATRIX (odd; 1-15 [5]) TO USE..: ",m
read "WHAT RANK GREY LEVEL (1..[13]) TO USE..... ",p
rankop n,n+1,m,p
 endif
 if answer eq 19
display n+1
read "WHAT SIZE MATRIX (1-255, [5]) TO USE : ",m
read "WHAT THRESHOLD LEVEL (1-255 [10]) TO USE : ",p
lowpnl n,n+1,m,p,_ON
 endif
 if answer eq 20
display n+1
read "WHAT SIZE MATRIX (1-255, [5]) TO USE : ",m
read "WHAT FACTOR ORIGINAL (+1[0.0]) TO USE : ",l
read "WHAT FACTOR LOWPASS (+1[1.0]) TO USE : ",o
read "HOW MANY REPS FOR LOWPASS [1] : ",p
lowpwt n,n+1,m,m,l,o,p
 endif
 if answer eq 21
display n+1
read "WHAT SIZE MATRIX (3-11, [3]) TO USE : ",m
gauss n,n+1,m
 endif
 endif
 if answer eq 3
clearmes
wrte "SELECT THE SHARPENING FILTER REQUIRED :"
wrte " 30 - DELEATE 31 - EMPHASIZE"
wrte " 32 - ENHANCE CONTOUR"
read "YOUR SELECTION IS : ",answer
 if answer eq 30
display n+1
read "DISCRIMINATE LEVEL (0-255, [10]) if automatic : ",m
read "WHAT SIZE (3,5,7 [5]) TO USE : ",p
delin n,n+1,m,p,_ON
 endif
 if answer eq 31
display n+1
read "WHAT SIZE MATRIX (1-255, [5]) TO USE : ",m
read "WHAT FACTOR ORIGINAL ( [10.0]) TO USE : ",l
read "WHAT FACTOR LOWPASS ( [9.5]) TO USE : ",o
read "WHAT grey OFFSET (-255..+255 [0]) TO USE : ",p
```

```
emphas n,n+1,m,m,l,o,p
 endif
 if answer eq 32
display n+1
read "TYPE OF CONTOUR ENHANCEMENT (2,3,5,7(3)): ",m
read "WHAT STRENGTH (1-9 [2]) TO USE : ",p
enhcont n,n+1,m,p
 endif
endif
if answer eq 4
clearmes
wrte "SELECT THE EDGES FILTER REQUIRED :"
wrte " 40 - SOBEL GRADIENT 41 - ROBERTS GRADIENT"
wrte " 42 - SUM GRADIENT  43 - MAX GRADIENT "
wrte " 44 - HIGHPASS      45 - LAPLACIAN    "
read "YOUR SELECTION IS : ",answer
 if answer eq 40
display n+1
sobel n,n+1
 endif
 if answer eq 41
display n+1
roberts n,n+1
 endif
if answer eq 42
display n+1
sumgrad n,n+1
 endif
 if answer eq 43
display n+1
maxgrad n,n+1
 endif
 if answer eq 44
display n+1
read "WHAT SIZE MATRIX (1-255, [5]) TO USE : ",m
read "CONTRAST ENHANCEMENT FACTOR (1.0..10.0(2.0)): ",l
read "POSITIVE OFFSET GREY LEVEL (0-255 [0])  : ",o
read "No. of iterations [1] TO USE : ",p
highpass n,n+1,m,m,l,o,p
 endif
 if answer eq 45
display n+1
read "TYPE OF LAPLACE FILTER (2,3,5,7(3)):  ",m
laplace n,n+1,m,_OFF,_OFF
 endif
endif
endmacro


###  IMAGE PROCESSING - GREY-LEVEL IMAGE ###

macro gipro
clearmes
wrte "Select NUMBER of GREY-LEVEL PROCESSING REQUIRED : "
wrte "  21  - TRANSFORM (will be a square image)"
wrte "  22  - SHIFT "
wrte "  23  - ROTATE  - fill in"
read "WHICH GEOMETRIC TRANSFORMATION DO YOU WANT TO USE
: ",answer
read "Your selection IS :  ",answer
read "WHICH IMAGE No. WOULD YOU LIKE TO USE  : ",n
read "What No. do you wish the OUTPUT IMAGER to be  :  ",a
wrte "YOU HAVE CHOSEN IMAGE NUMBER      : ",n
if answer eq 21
read "What is the reference image  : "
transform
n,l,a,6,256,256,0.00,1.00,1.00,0.0,_ON
endif
if answer eq 22
invert n,a
endif
if answer eq 23
rotate n,a,
endif
endmacro


###  HISTOGRAMS  ###

macro hispr
clearmes
read "What image No. do you wish to use   :  ",n
display n
wrte " You have chose image No.   :  ",n
wrte "Select the NUMBER of the histogram function required"
wrte " 1  -  Histogram in the overlay;              "
wrte " 2  -  on the data monitor with stats;"
wrte " 3  -  Object (inter.) OR 4 - profile 3D (GREY LEVEL)"
read "Your selection IS       : ",answer
if answer eq 1
 histovl n,-1.0,_OFF
endif
if answer eq 2
 histogram n,_OFF,_OFF
endif
if answer eq 3
 objhist n,1.2,_ON
endif
if answer eq 4
 prof3d n,0,0,100,100,4,30.0,0,_ON
endif
endmacro


###  IDENTIFICATION  ###

macro ident
clearmes
read "WHICH IMAGE WOULD YOU LIKE TO USE          ",n
display n
wrte "YOU HAVE CHOSEN IMAGE NUMBER          ",n
read "What No. do you wish the OUTPUT IMAGE TO BE:  ",a
 loadlut "grey"
identframe n,a,_ON,1.652,450,30,30,_ON,_OFF,_OFF
wrte "Choose the size of the exclusion on the size of the"
wrte "object to be measured, i.e. 0.5 X MO (20p)"
endmacro


###  IMAGE CAPTURE  ###

macro imcap
clearmes
tvchan 0,0
clear 1,0
wrte "The B/W camera is now ready to be turned on"
clovl 1
pause
tvon
wrte " ONCE THE IMAGE IS READY, PRESS ANY KEY TO CAPTURE
IMAGE"
pause
grab 1,6,_OFF,0
wrte "an image has now been captured....."
display 2
add 1,2,2,3
display 4
add 3,4,4,3
display 4
add 5,6,6,3
display 4
add 2,4,4,3
display 6
add 2,6,6,3
clear 1,0
display 1
add 4,6,1,3
wrte "The captured image has been improved by addition....."
display 1
endmacro


###  IMAGE STORAGE AND RETRIEVAL VERSION ONE  ###

macro imsr_1
clearmes
mogrtxt1=mogrtxt2=""
wrte "If you wish to retrieve (1) an image"
read "or store (2) an image type :  ",answer
if answer eq 1
read " What No. of IMAGE  do you wish this to be :",n
 display n
 wrte "Type in the name of the image you wish to"
read "retrieve :    ",mogrtxt1
 display n
 getim mogrtxt1,n
wrte "YOU HAVE RETRIEVED IMAGE   : ",mogrtxt1
getimhead mogrtxt1,mogrtxt2
Gatring 1,0,0,mogrtxt2,-1,0,0
 wrte "TEXT SAVED WITH IMAGE IS : ",mogrtxt2
endif
if answer eq 2
 read "Type in the No. of the image you want to store  : ",n
 display n
 wrte "You have chosen image     :  ",n
 read " Type in IMAGE name you wish to store",mogrtxt1
  wait 500
  storim mogrtxt1,n
 read " Type an image HEADER SAVED WITH IMAGE",mogrtxt2
 setimhead mogrtxt1,mogrtxt2
 wrte "This is what is saved with the image:  ",mogrtxt2
endif
endmacro


###  IMAGE STORAGE AND RETRIEVAL VERSION TWO  ###

macro imsr_2


### IMAGE RETRIEVAL - MANUAL ###

wrte "HAVE THE F: DRIVE READY FOR IMAGE RETRIEVAL"
ciall
clearmes
read "No. of image 1 is :  ",m
read "No. of image 2 is :  ",n
read "No. of image 3 is :  ",o
display 1
getim m,1
display 2
getim n,2
display 3
getim o,3
wrte "IMAGE no :  ",m," has been retrieved to :  1 "
wrte "IMAGE no :  ",n," has been retrieved to :  2 "
wrte "IMAGE no :  ",o," has been retrieved to :  3 "
wrte "Retrieved imageS to be SEGMENTED MANUALLY"


### IMAGE RETRIEVAL - MANUAL WITH EDITING ###

wrte "HAVE THE F: DRIVE READY FOR IMAGE RETRIEVAL"
ciall
clearmes
loadlut "ident"
read "No. of image 1 is :  ",m
read "No. of image 2 is :  ",n
display 1
getim m,1
display 4
getim n,4
wrte "IMAGE no :  ",m," has been retrieved to :  1 "
wrte "IMAGE no :  ",n," has been retrieved to :  4 "
wrte "Retrieved imageS to be SEGMENTED MANUALLY"


### IMAGE STORAGE ###

clearmes
for o=1, o<7, o=o+1
display o
wrte "The No. of the image you will store  :  ",o
wrte "The image will be stored as      :  ",mogrtxt2 + string(m)
storim mogrtxt2 + string(m),o
wrte "Image header saved with image is:  ",mogrtxt3
wrte " the end name of the image stored is : ",m
setimhead mogrtxt2 + string(m),mogrtxt3
m=m+1
endfor
endmacro


### IMAGE STORAGE ###

wrte " store 3 imageS :  "
wrte "The No. of the image you will store  :  ",m
storim m,4
wrte "The No. of the image you will store  :  ",n
storim n,5
wrte "The No. of the image you will store  :  ",o
storim o,6
wrte " The end of storing images."
ciall
endfor
endfor
stop


###  MEASUREMENT FUNCTIONS  ###

macro measf
clearmes
read "WHICH IMAGE DO YOU WISH TO MEASURE.....",n
display n
wrte ""
wrte "USING IMAGE No.   ",n,"   FOR MEASUREMENT"
# DENSITOMETRIC PARAMETERS
while 1
 loadlut "grey"
 Measd n
 if(_STATUS) : break
 DBappend "MOGR-DB1"
endwhile
Dbclose "MOGR-DB1"
# FIELDBASED PARAMETERS
identify n,n+1,_ON,_OFF
display n+1
Measf n+1
Dbappend "MOGR-DB2"
Dbclose "MOGR-DB2"
# OBJECTSPECIFIC PARAMETERS
identify n,n+1,_ON,_OFF
display n+1
while 1
 Measo n+1
 if(_STATUS) : break
 Dbappend "MOGR-DB3"
endwhile
Dbclose "MOGR-DB3"
# OBJECTSPECIFIC PARAMETERS
identify n,n+1,_ON,_OFF
display n+1
while 1
 Measo n+1
 if(_STATUS) : break
 Dbappend "MOGR-DB4"
endwhile
Dbclose "MOGR-DB4"
wrte "..............the measurement is completed."
endmacro
```

```
### USER MEASUREMENT AND DATA BASE PARAMETERS VERSION TWO ###

macro medbp_2
clearmes
text1="co"
text2="ro"
text3="fs"
text4="fh"
text5="all"
text21="mgfield"
text11="mgfeat1"
text12="mgfeat2"
text13="mgfeat3"
text14="mgfeat4"
text15="mgfeat5"
read "DATA BASE TO USE FOR field RESULTS (11)",text21
read "DATA BASE TO USE FOR RESULTS of cocci (1)",text1
read "DATA BASE FOR RESULTS of rods (2)",text2
read "DATA BASE FOR RESULTS of fungal spore (3)",text3
read "DATA BASE FOR RESULTS of fungal hypha (4)",text4
read "DATA BASE FOR RESULTS of rest of things (5)",text5
write "will use ",text1," as DATA BASE for cocci (1)"
write "will use ",text2," as DATA BASE for rods (2)"
write "will use ",text3," as DATA BASE for fungal spores (3)"
write "will use ",text4," as DATA BASE for fungal hypha (4)"
write "will use ",text5," as DATA BASE for rest of things (5)"
write "INITIALIZE THE PARAMETERS (1)"
# FIELDBASED PARAMETERS
InitField TOTALAREA, REFAREA, AREAP, FIELDCOUNT
TOTALAREA = REFAREA = AREAP = FIELDCOUNT = 0
mgfeat21[]=TOTALAREA, AREAP, FIELDCOUNT
Oberase text21
DBcreate text21,"mgfeat21"
Dbopen text21,"mgfeat21"
write text21,"mgfeat21"
# OBJECTSPECIFIC PARAMETERS
InitObj AREA,DMAX,DMIN,FCIRCLE,ACPX,ACPY
AREA = DMAX = DMIN = FCIRCLE = CGRAVY = CGRAVX = ACPX = ACPY = 0
global ELONG
ELONG = 0.0
mgfeat1[]=AREA,FCIRCLE,ELONG
mgfeat2[]=AREA,FCIRCLE,ELONG
mgfeat3[]=AREA,FCIRCLE,ELONG
mgfeat4[]=AREA,FCIRCLE,ELONG
mgfeat5[]=AREA,FCIRCLE,ELONG
for i=1, i<2, i=i+1
   name1=text1
   name2=text11
   Oberase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
   name1=text2
   name2=text12
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
   name1=text3
   name2=text13
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
   name1=text4
   name2=text14
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
  name1=text5
  name2=text15
  DBerase name1
  DBcreate name1,name2
  DBopen name1,name2
  write name1, name2
  DBclose name1
endfor
write "NOW SETTING THE 2ND DATA BASES UP - FOR THE ADDITION OF FIELD NUMBER"
DBerase "co-2","mgfeat6"
DBerase "ro-2","mgfeat7"
DBerase "fs-2","mgfeat8"
DBerase "fh-2","mgfeat9"
DBerase "all-2","mgfeat10"
wait 500
external AREA,FCIRCLE
global ELONG,USERNO
AREA = FCIRCLE = 0
ELONG = USERNO = 0
mgfeat6[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat7[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat8[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat9[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat10[]=AREA,FCIRCLE,ELONG,USERNO
wait 500
DBcreate "co-2","mgfeat6"
DBclose "co-2"
DBcreate "ro-2","mgfeat7"
DBclose "ro-2"
DBcreate "fs-2","mgfeat8"
DBclose "fs-2"
DBcreate "fh-2","mgfeat9"
DBclose "fh-2"
DBcreate "all-2","mgfeat10"
DBclose "all-2"
endif
endmacro


### USER MEASUREMENT AND DATA BASE PARAMETERS VERSION ONE ###

macro medbp_1
clearmes
write "DO YOU WISH TO INITIALIZE THE PARAMETERS (1)"
write "                    OR  "
read " JUST OPEN THE RELEVANT DATA BASES (2).....",answer
if answer eq 1
DBerase "MOGR-DB1", "myfeat1"
DBerase "MOGR-DB2", "myfeat2"
DBerase "MOGR-DB3", "myfeat3"
DBerase "MOGR-DB4", "myfeat4"
DBerase "MOGR-DB5", "myfeat5"
# DENSITOMETRIC PARAMETERS
InitGrey GREYM, GREYSD
GREYM = GREYSD = 0.00
mgfeat1[]=GREYM, GREYSD
# FIELDBASED PARAMETERS
InitField TOTALAREA, AREAP, FIELDCOUNT
TOTALAREA = AREAP = FIELDCOUNT = 0
mgfeat2[]=TOTALAREA, AREAP, FIELDCOUNT
# OBJECTSPECIFIC PARAMETERS
InitObjANGLEDMAX,ANGLEDMIN,AREA,DCIRCLE,DMAX,DMIN
InitObj FCIRCLE,FSHAPE,PERIM
ANGLEDMAX = ANGLEDMIN = AREA = DCIRCLE = DMAX = DMIN = 0.00
FCIRCLE = FSHAPE = PERIM = CGRAVY = CGRAVX = 0.00
mgfeat3[]=ANGLEDMAX,ANGLEDMIN,AREA,DCIRCLE,DMAX,DMIN
mgfeat4[]=FCIRCLE,FSHAPE,PERIM
DBcreate "MOGR-DB1", "mgfeat1"
DBcreate "MOGR-DB2", "mgfeat2"
DBcreate "MOGR-DB3", "mgfeat3"
DBcreate "MOGR-DB4", "mgfeat4"
endif
if answer eq 2
DBopen "MOGR-DB1", "mgfeat1"
DBopen "MOGR-DB2", "mgfeat2"
DBopen "MOGR-DB3", "mgfeat3"
DBopen "MOGR-DB4", "mgfeat4"
endif
endmacro


### PARTIAL INITIALIZATION ###

macro peel
clall
clearmes
loadlut "grey"
answer=1
endmacro


### RESULTS VERSION ONE ###

macro resr_1
clearmes
mogrtxt4="ro"
write " WHAT RESULTS DO YOU WISH TO SEE ....."
write "  select : ro   ;co      ;fh      ;fs     ;all"
write "  select : ro-2 ;co-2    ;fh-2    ;fs-2   ;all-2  "
read "select :",mogrtxt4
outlist mogrtxt4_OFF
endmacro


### RESULTS VERSION TWO ###

macro resul_2
clearmes
write "Select how RESULTS are to be DISPLAYED "
write "  1 - LIST     2 - histogram"
write "  3 - SCREEN   4 - scatergram"
write "  4 - distrogram"
write "  5 - cross    6 - prob"
read " YOUR SELECTION is  :  ",answer
mogrtxt1="MOGR-DB1"
mogrtxt2="AREA"
clearmes
if answer eq 1
read " Which DB (MOGR-DB*) to use (1-4)  :  ",mogrtxt1
outlist mogrtxt1,_OFF
stop
endif
if answer eq 2
read " Which DB do you wish to use (1-4)  :  ",mogrtxt1
read "What parameter do you wish to use?  ",mogrtxt2
outhist mogrtxt1,mogrtxt2,15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
stop
endif
if answer eq 3
loadlut "gold"
display 1
DBopen "MOGR-DB5","myfeat5"
i=1
while 1
  DBread "MOGR-DB5"
  if(_STATUS) : break
  Gatring 1, int (CGRAVX),int (CGRAVY),i, 255,0,0
  i=i+1
endwhile
endif
if answer eq 4
mogrtxt1=mogrtxt2="MOGR-DB3"
read "  DB for 1st parameter (1-4)  :  ",mogrtxt1
read "  DB for 2nd parameter (1-4)  :  ",mogrtxt2
mogrtxt3=mogrtxt4="DMAX"
read "    1st parameter?    :  ",mogrtxt3
read "    2nd parameter?    :  ",mogrtxt4
outscatmogrtxt1,mogrtxt3,mogrtxt2,mogrtxt4,_OFF,_OFF,_ON,_ON,0,0,0,0
endif
if answer eq 5
outdistmogrtxt1,mogrtxt2,mogrtxt3,mogrtxt4,10,10,_ON,_ON,0.00,100.00,0.00,100.00
endif
if answer eq 6
outcrossmogrtxt1,mogrtxt2,mogrtxt3,15,_OFF,_OFF,0,0,_ON,0.00,100.00,0.00,100.00
endif
if answer eq 7
outprobmogrtxt1,mogrtxt2,mogrtxt3,15,_OFF,0,_ON,0.00,100.00
endif
endif
endmacro


### RESULTS VERSION THREE ###

macro resr_3
loadlut "grey"
i=4
name1=text1 + string(i)
name2=text2 + string(i)
DBopen name1,name2
while 1
  DBread name1
  if _STATUS break
Gatring 4,int(CGRAVX),int(CGRAVY),int(AREA),-1,0,0
endwhile
DBclose name1
endmacro


### SCALE FUNCTIONS VERSION TWO ###

macro scale_2
clearmes
write " Use an already calibrated scale (2)"
wait 1000
write "IMAGE  :  ",name1,"    IS NOW OVERLAID WITH SCALE  "
if i == 1
scalgeom 1,"CZ682"_OFF_OFF
endif
if i == 2
scalgeom 1,"CO3B5"_OFF_OFF
endif
if i == 3
scalgeom 1,"CZ6B4"_OFF_OFF
endif
wait 1000
marker 1,1,1,_ON,_ON,_ON,2
wait 1000
endmacro


### SCALE FUNCTIONS VERSION ONE ###

macro scale_1
clearmes
write "Do you wish to interactively set the scale (1)"
write " or use an already calibrated scale (2)"
read "Type 1 or 2 to set the scale.",answer
if answer eq 1
 write "turn on the TV/VCR"
 pause
 tvon
 pause
 tvinp 1
 tvon
 pause
 tvinp 2
 tvoff
 display 3
 add 1,2,3,3
 tvchan 0,0
 display 3
 scalgeom 3,"1:1-scale"_ON,_ON
 marker 3,10,10,_ON,_ON,_ON,2
endif
if answer eq 2
display 1
scalgeom 1,"mag=20x"_OFF,_ON
marker 1,10,10,_ON,_ON,_ON,2
endif
endmacro


### SCALE FUNCTIONS VERSION THREE ###

macro scale_3
clearmes
write " Use an already calibrated scale (2)"
wait 1000
n=1
write "IMAGE NUMBER  :  ",n," IS NOW OVERLAID WITH SCALE  "
display n
scalgeom n,"CZ6B5"_OFF_OFF
wait 1000
marker n,1,1,_ON,_ON,_ON,2
wait 1000
endmacro
```

```
### SEGMENTATION VERSION ONE ###

macro seg_1
clearmes
write "Select NUMBER of SEGMENTATION function"
write "  1 - ....with multiple phases;   "
write "  2 - ....with reference image;   "
write "  3 - ....at 2 threshold levels;   "
read "Your selection IS :  ",answer
read "WHICH IMAGE No. WOULD YOU LIKE TO USE      ",n
read "What No. do you wish the OUTPUT IMAGER to be      ",a
write "YOU HAVE CHOSEN IMAGE NUMBER      ",n
if answer eq 1
 display n
read "What No. of phases do you want discriminated: ",p
write " YOU HAVE SELECTED  ",p," PHASES TO BE DISCRIMINATED"
disaut n,a,p,_OFF,_ON,_ON,5,1,_ON,_ON
write "This is an automatic discrimination -"
write " with a no. of phases  =  ",p
endif
if answer eq 2
 display n
read "What is the No. of the reference IMAGE      ",p
write "You have chosen this image as a REF image"
 display p
pause
disref n,p,a,2,_OFF,_OFF,_ON
display n
endif
if answer eq 3
 display n
dis2lev n,a,120,90,_OFF,_OFF,1
endif
endmacro


### SEGMENTATION VERSION TWO ###

macro seg_2
for a=1,a<3,a=a+1
clearmes
write " SEGMENTATION function"
write "  3 - ....at 2 threshold levels;   "
display 1
dis2lev 1,2,0,160,_ON,_ON,1
display 2
contour 2,2,3,7,128,_ON,_ON
copyovl 3,1
outlink 1,3,1,9,255,_OFF
dis2lev 3,3,255,255,_ON,_OFF,1
combine 2,3,1,0,0.5,0.5
clovl 1
scrap 1,1,_OFF,0,100,_ON,_ON
display 4
dis2lev 4,5,0,160,_ON,_ON,1
display 5
contour 5,5,6,7,128,_ON,_ON
copyovl 6,4
outlink 4,6,4,9,255,_OFF
dis2lev 6,6,255,255,_ON,_OFF,1
combine 5,6,4,0,0.5,0.5
clovl 4
scrap 4,4,_OFF,0,100,_ON,_ON
display 4
endmacro


### FULL SYSTEM INITIALIZATION ###

macro setup
clearall
clearallov
clall
clearmes
messatop
resetpar
resetlim
alpha
setimpath "f:/BTD/MO-GR1/images"
setframe "F766"
loadlut "grey"
ovcolour 3
clear 1,0
tvchan 0,0
setuplist
mogrtxt1=mogrtxt2=mogrtxt3=mogrtxt4=""
a=p=n=i=o=m=1
answer=1
col=1
display n
setuplist
endmacro
```

```
### MEASUREMENT AND DATA BASE PARAMETERS VERSION ONE
###

macro smdbp_1
col = 1
identify 2,3
display 3
text1="co"
text2="ro"
text3="fs"
text4="fh"
text5="all"
text21="mgfield"
text11="mgfeat1"
text12="mgfeat2"
text13="mgfeat3"
text14="mgfeat4"
text15="mgfeat5"
wait 10000
# FIELDBASED PARAMETERS
DBopen text21,"mgfield"
wait 10000
display 2
identify 2,2
Measf 2
DBappend text21
write "No. of bacteria in image  =  ",FIELDCOUNT
DBclose text21
# OBJECTSPECIFIC PARAMETERS
for i=1, i<2, i=i+1
name1=text1
name2=text11
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text2
name2=text12
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text3
name2=text13
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text4
name2=text14
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text5
name2=text15
DBopen name1,name2
endfor
Gatring 2,50,50,"red = cocci; yellow = rod",-1,0,0
Gatring 2,50,70,"blue = fungal spore; pink = fungal hypha",-1,0,0
wait 10000
display 3
while (1)
   Messo 3
   if (_STATUS) : break
   ELONG=DMAX/DMIN
if int(ELONG)<1.2 && int(FCIRCLE)>=0.7 && int(AREA)<0.1  :col=25
if int(ELONG)<1.2 && int(FCIRCLE)>=0.7 && int(AREA)<0.1  :DBappend text1
if int(AREA)<0.1 && int(ELONG)<=3 && int(FCIRCLE)<0.6  :col = 16
if int(AREA)<0.1 && int(ELONG)<=3 && int(FCIRCLE)<0.6  :DBappend text2
if int(ELONG)<3 && int(AREA)>0.5  :col=233
if int(ELONG)<3 && int(AREA)>0.5  :DBappend text3
if int(AREA)>0.1 && int(ELONG)>=3  :col=91
if int(AREA)>0.1 && int(ELONG)>=3  :DBappend text4
if int(AREA)>0  : col=3
if int(AREA)>0  : DBappend text5
endif
wait 500
x=int(ACPX)
y=int(ACPY)
Grectg 2,x,y,15,15,col,7
Gatring 2,x,y,int(AREA*100),-1,1,0
endwhile
DBclose text1
DBclose text2
DBclose text3
DBclose text4
DBclose text5
write "..............the measurement  is completed."
endmacro
```

```
### MEASUREMENT AND DATA BASE PARAMETERS VERSION TWO
###

macro smdbp_2
for n=1,n<7,n=n+1
n=1
write "                  THE CURRENT VALUE OF N IS    ",n
loadlut "ident"
col = 1
display n
text1="co"
text2="ro"
text3="fs"
text4="fh"
text5="all"
text21="mgfield"
text11="mgfeat1"
text12="mgfeat2"
text13="mgfeat3"
text14="mgfeat4"
text15="mgfeat5"
write "USING IMAGE No.   ",n,"   FOR MEASUREMENT"


# FIELDBASED PARAMETERS

DBopen text21,"mgfield"
Measf n
DBappend text21
write "No. of bacteria in image",n," =  ",FIELDCOUNT
DBclose text21


# OBJECTSPECIFIC PARAMETERS

for i=1, i<2, i=i+1
name1=text1
name2=text11
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text2
name2=text12
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text3
name2=text13
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text4
name2=text14
DBopen name1,name2
endfor
for i=1, i<2, i=i+1
name1=text5
name2=text15
DBopen name1,name2
endfor
Gatring n,0,0,"1    = CO",-1,0,0
Grectg n,0,0,100,100,-1,0
Gatring n,0,100,"2    = RO",-1,0,0
Grectg n,0,100,100,100,-1,0
Gatring n,0,200,"3    = FS",-1,0,0
Grectg n,0,200,100,100,-1,0
Gatring n,0,300,"4    = FH",-1,0,0
Grectg n,0,300,100,100,-1,0
Gatring n,0,400,"5    = OTHER",-1,0,0
Grectg n,0,400,100,100,-1,0
Gatring n,10,500,"AREA (COLOUR); ELONGATION THEN FCIRCLE",-1,0,0
write " HIT <ESC> TO STOP COUNTING"
write "  What select object to measure (white), then classify!! "
display n
while (1)
   Goursor n,x,y,1
   if _STATUS eq 27: break
   Messo n,x,y
   ELONG=DMAX/DMIN
    Goursor n,x,y,1
  if (x >=0 && x <=100 && y >=0 && y <=100) : DBwnte text1
   endif
   if (x >=0 && x <=100 && y >=100 && y <=200) : DBwnte text2
   endif
   if (x >=0 && x <=100 && y >=200 && y <=300) : DBwnte text3
   endif
   if (x >=0 && x <=100 && y >=300 && y <=400) : DBwnte text4
   endif
   if (x >=0 && x <=100 && y >=400 && y <=500) : DBwnte text5
   endif
x=int(ACPX)
y=int(ACPY)
Grectg n,x,y-25,15,15,col,7
Gatring n,x,y,int(AREA*1000),-1,1,0
Gatring n,x,y+25,int(ELONG*100),-1,1,0
Gatring n,x,y+50,int(FCIRCLE*100),-1,1,0
endwhile
DBclose text1
DBclose text2
DBclose text3
DBclose text4
DBclose text5
write "..............the measurement  is completed."
endmacro
```

```
### ZOOM FUNCTIONS ###

macro zoom
clearmes
read " What image No. do you wish to use      ",n
display n
write "YOU HAVE CHOSEN IMAGE No. : ", n ," FOR ZOOM"
write "Select the NUMBER of the ZOOM function required"
write "  1 - ZOOM UP;   "
write "  2 - ZOOM DOWN;  "
write "  3 - SCROLL;     "
read "Your selection IS :  ",answer
if answer eq 1
 display n+1
 zoomup n,n+1,0,0,0,_ON
endif
if answer eq 2
 display n+1
 zoomdn n,n+1,0,0,0,_ON
endif
if answer eq 3
 scroll
endif
endmacro


### USER MEASUREMENT AND DATA BASE PARAMETERS ###

macro medbp.3
clearmes
text1="co"
text2="ro"
text3="fs"
text4="fh"
text5="all"
text21="mgfield"
text11="mgfeat1"
text12="mgfeat2"
text13="mgfeat3"
text14="mgfeat4"
text15="mgfeat5"
read "DATA BASE TO USE FOR field RESULTS (11)",text21
read "DATA BASE TO USE FOR RESULTS of cocci (1)",text1
read "DATA BASE FOR RESULTS of rods (2)",text2
read "DATA BASE FOR RESULTS of fungal spore (3)",text3
read "DATA BASE FOR RESULTS of fungal hyphe (4)",text4
read "DATA BASE FOR RESULTS of rest of things (5)",text5
write "will use ",text1," as DATA BASE for cocci (1)"
write "will use ",text2," as DATA BASE for rods (2)"
write "will use ",text3," as DATA BASE for fungal spores (3)"
write "will use ",text4," as DATA BASE for fungal hyphe (4)"
write "will use ",text5," as DATA BASE for rest of things (5)"
write "DO YOU WISH TO INITIALIZE THE PARAMETERS (1)   OR"
read " JUST OPEN THE RELEVANT DATA BASES (2).....",answer
if answer eq 1


# FIELDBASED PARAMETERS

IntField TOTALAREA, REFAREA, AREAP, FIELDCOUNT
TOTALAREA = REFAREA = AREAP = FIELDCOUNT = 0
mgfeat21[]=TOTALAREA, AREAP, FIELDCOUNT
DBerase text21
DBcreate text21,"mgfeat21"
DBopen text21,"mgfeat21"
write text21,"mgfeat21"
# OBJECTSPECIFIC PARAMETERS
IntObj AREA,DMAX,DMIN,FCIRCLE,ACPX,ACPY
AREA = DMAX = DMIN = FCIRCLE = CGRAVY = CGRAVX = ACPX = ACPY
= 0
global ELONG
ELONG = 0.0
mgfeat1[]=AREA,FCIRCLE,ELONG
mgfeat2[]=AREA,FCIRCLE,ELONG
mgfeat3[]=AREA,FCIRCLE,ELONG
mgfeat4[]=AREA,FCIRCLE,ELONG
mgfeat5[]=AREA,FCIRCLE,ELONG
for i=1, i<2, i=i+1
   name1=text1
   name2=text11
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
   name1=text2
   name2=text12
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
   name1=text3
   name2=text13
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
   name1=text4
   name2=text14
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
for i=1, i<2, i=i+1
   name1=text5
   name2=text15
   DBerase name1
   DBcreate name1,name2
   DBopen name1,name2
   write name1, name2
   DBclose name1
endfor
write "NOW SETTING THE 2ND DATA BASES UP - FOR THE ADDITION OF
FIELD NUMBER"
DBerase "co-2","mgfeat6"
DBerase "ro-2","mgfeat7"
DBerase "fs-2","mgfeat8"
DBerase "fh-2","mgfeat9"
DBerase "all-2","mgfeat10"
wait 500
external AREA,FCIRCLE
global ELONG,USERNO
AREA = FCIRCLE = 0
ELONG = USERNO = 0
mgfeat6[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat7[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat8[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat9[]=AREA,FCIRCLE,ELONG,USERNO
mgfeat10[]=AREA,FCIRCLE,ELONG,USERNO
wait 500
DBcreate "co-2","mgfeat6"
DBclose "co-2"
DBcreate "ro-2","mgfeat7"
DBclose "ro-2"
DBcreate "fs-2","mgfeat8"
DBclose "fs-2"
DBcreate "fh-2","mgfeat9"
DBclose "fh-2"
DBcreate "all-2","mgfeat10"
DBclose "all-2"
endif

if answer eq 2
text1="co"
text2="ro"
text3="fs"
text4="fh"
text5="all"
text21="mgfield"
text11="mgfeat1"
text12="mgfeat2"
text13="mgfeat3"
text14="mgfeat4"
text15="mgfeat5"
write "will use ",text21," as DATA BASE for field (11)"
write "will use ",text1," as DATA BASE for cocci (1)"
write "will use ",text2," as DATA BASE for rods (2)"
write "will use ",text3," as DATA BASE for fungal spores (3)"
write "will use ",text4," as DATA BASE for fungal hyphe (4)"
write "will use ",text5," as DATA BASE for rest of objects(5)"
DBopen text21,"mgfeat21"
for i=1, i<2, i=i+1
   name1=text1 # + string(i)
   name2=text11 # + string(i)
   DBopen name1,name2
   write name1, name2
endfor
for i=1, i<2, i=i+1
   name1=text2 # + string(i)
   name2=text12 # + string(i)
   DBopen name1,name2
   write name1, name2
endfor
for i=1, i<2, i=i+1
   name1=text3 # + string(i)
   name2=text13 # + string(i)
   DBopen name1,name2
   write name1, name2
endfor
for i=1, i<2, i=i+1
   name1=text4 # + string(i)
   name2=text14 # + string(i)
   DBopen name1,name2
   write name1, name2
endfor
for i=1, i<2, i=i+1
   name1=text5
   name2=text15
   DBopen name1,name2
   write name1, name2
endfor
DBclose "co"
DBclose "ro"
DBclose "fs"
DBclose "fh"
DBclose "all"
DBopen "co-2","mgfeat1"
DBopen "ro-2","mgfeat2"
DBopen "fs-2","mgfeat3"
DBopen "fh-2","mgfeat4"
DBopen "all-2","mgfeat5"
endif
endmacro


### IMAGE RETRIEVAL ###

macro imar.3
mogrtxt2 = "c19-"
loadfut "GREY"
dall
clearmes
write "Retrieve an image"
n=1
display n
for i=1,i<3,i=i+0
getim mogrtxt2 + string(i),n
n=n+3
write "YOU HAVE RETRIEVED IMAGE :  ",mogrtxt2 + string(i)," TO  ",n-3
i=i+1
endfor
write  "TWO DIFFERENT, CONSECUTIVE IMAGES HAVE BEEN
RETRIEVED"
endmacro
```

APPENDIX FOUR : IMAGE ANALYSIS STAND ALONE PROGRAMMES

```
##################################################
# MACRO          # BACKGROUND CORRECTION
#                #
# TITLE          # MULTIPLE IMAGE BACKGROUND CORRECTION
#                #
# DESCRIPTION    # MULTIPLE RETRIEVAL, BACKGROUND
#                # CORRECTION AND STORAGE OF THE NEW IMAGE.
#                #
#                # DATE WRITTEN   # 23/09/93
#                #
# SAVED AS       # 1MO-BKCO.(mcr)
#                #
##################################################

macro setup
endmacro

###  LIST OF MACROS OPERATIONAL  ###
setup          # INITIALIZATION
bgcor          # BACKGROUND CORRECTION
imar           # IMAGE STORAGE & RETRIEVAL
filtr          # FILTER ENHANCEMENT
stop


# INITIALIZATION

macro setup
clearall
clearallov
clall
clearmes
messatop
resetper
resetlim
alpha
setimpath "f:/bf-60h-n/images"
setframe "F768"
loadlut "grey"
ovcolour 3
clear 1,0
display 1
tvchan 0,0
monsync "CO"
clearmes
answer=0
mogrtxt1=mogrtxt2=mogrtxt3=mogrtxt4=""
a=p=n=l=o=m=1
display n
setuplist
endmacro


# CONTINUOUS IMAGE RETRIEVAL

clearmes
mogrtxt2 = ""
clovl 1
clear 1,0
write "HAVE THE F: DRIVE READY FOR IMAGE RETRIEVAL"
for l=43, l<50, l=l+1
clall
getim mogrtxt2 + string (l)
write mogrtxt2 + string (l)
write "Retrieved image to be improved by background correction"


# BACKGROUND CORRECTION

display 2
linklist 1,2
write "CREATE (1) a new BG img"
write "IT IS SET FOR light OBJ ON A dark BACKGROUND"
display 3
trackgr 2,3,50,100,_OFF
shaddef 3,3
write "The shading correction is set to additive (ILLUMINATION)"
display 4
shadcorr 2,4,3,_ON,0


# FILTERS

write "MACRO USED FOR IMAGE ENHANCEMENT - FILTERS"
write "YOU HAVE CHOSEN IMAGE No.  ",3,"  TO BE ENHANCED"
display 5
enhcont 4,5,7,2


# IMAGE STORAGE

write " store (4) an image :  "
write "The No. of the image you will store  :  ",mogrtxt2 + string(l)
storim mogrtxt2 + string(l),5
clearmes
clall
endfor
stop

#####################################
write "     THIS IS THE END OF THE MACRO "
write "     CALLED  ***  1MO-bkco.MCR  ***  "
#####################################

#####################################
# MACRO                    # AUTOMATIC PROCESSING
#                          #
# TITLE                    # MULTIPLE AUTOMATIC IMAGE
#                          # PROCESSING
#                          #
# DESCRIPTION              # LARGE SCALE AUTOMATIC
                           # PROCESSING OF
#                          # IMAGES
#                          #
# DATE  WRITTEN            # 19/07/93
#                          #
# SAVED AS       # 1MO-PRO (mcr)
#                          #
#####################################

macro pro
endmacro

###  LIST OF MACROS OPERATIONAL  ###
setup          # FULL INITIALIZATION
pro            # PROCESSING
stop

setimpath "f:/bld/mo-gr1/stats/images"
write "HAVE THE F: DRIVE READY FOR IMAGE RETRIEVAL"
mogrtxt2 = "c30-"
for l=45, l<70, l=l+1
clall
clearmes
display 1
write "      RETRIEVING     ",mogrtxt2 + string(l),"    TO IMAGE 1"
loadlut "stair"
getim mogrtxt2 + string(l),1
display 2
write "Retrieved image to be SCRAPPED AUTOMATICALLY"
loadlut "rainbow"
copywind 1,2,0,0,383,512,0,0,3,_OFF
copywind 1,3,383,0,383,512,384,0,3,_OFF
scrap 2,4,_OFF,0,50,_ON,_ON
scrap 3,5,_OFF,0,50,_ON,_ON
display 6
add 4,5,6,2
display 2
linklin 6,2,1,1
write "SCRAPPED IMAGE NOW IDENTIFIED IN FIELD MODE"
display 3
identframe 2,3,_ON,4,766,510,1,1,_ON,_OFF,_ON
display 4
fill 3,4
loadlut "spectra"
identify 4,5,_ON,_OFF
display 5


# IMAGE STORAGE

clearmes
write "The image (1) will be stored as  :  ",mogrtxt2 + string(l)
storim mogrtxt2 + string(l),5
endfor
stop
#####################################
write "     THIS IS THE END OF THE MACRO "
write "     CALLED  ***  1MO-PRO.MCR  ***  "
#####################################


##########################################
#                          #
# MACRO                    # IMAGE ENHANCEMENT
#                          #
# TITLE                    # IMAGE ADDITION
#                          #
# DESCRIPTION              # LARGE SCALE ADDITION,
#                          # RETRIEVAL
#                          # AND STORAGE OF IMAGES
#                          #
# DATE  WRITTEN            # 13/06/93
#                          #
# SAVED AS                 # 1MO-IMAD.(mcr)
#                          #
##########################################

macro imcap
endmacro

###  LIST OF MACROS OPERATIONAL  ###
setup          # INITIALIZATION
imcap          # IMAGE CAPTURE
mar 2          # IMAGE STORAGE & RETRIEVAL
stop

mogrtxt2 = "c30-"
read "The NAME of the image you will store  :  ",mogrtxt2
setimhead m,"biofilm 2, 21/09/93, after 60 hrs, 10MM hexanoic acid, 30oC"
mogrtxt3 = "c30-" img;100x,z,26/09/93,10  days old;DIC"
read " Image HEADER SAVED WITH IMAGE = ",mogrtxt3
setimpath "f:/bld/camu-30"


# CONTINUOUS IMAGE RETRIEVAL, ADDITION AND STORAGE

clearmes
clearallov
clearall
write "HAVE THE F: DRIVE READY FOR IMAGE RETRIEVAL"
for l=7, l<66, l=l+1
clearmes
clearallov
clearall 0
for l=29, l<101, l=l+1
 for o=1, o<7, o=o+1
 Gstring o,100,200,mogrtxt2 + string(l),-1,1,0
 getim mogrtxt2 + string(l),o
 getimhead mogrtxt2 + string(l),mogrtxt3
 display o
 write mogrtxt3
 Gstring o,100,100,mogrtxt3,-1,1,0
 write "IMAGE no  :  ",mogrtxt2 + string(l),"  has been retrieved to  :  ",o
 l=l+1
 endfor
endfor

write "an image has now been RETRIEVED 6X, number  :  "
wait 100000
display 2
add 1,2,2,3
wait 100000
display 4
add 3,4,4,3
display 4
add 5,6,6,3
wait 100000
display 4
add 2,4,4,3
display 6
add 2,6,6,3
clear 1,0
display 1
add 4,6,1,3
wait 100000
write "The captured image has been improved by addition....."
display 1


# IMAGE STORAGE

clearmes
write " store (2) an image :  "
write "The No. of the image you will store  :  "
wait 100000
storim l,1
write " Image HEADER SAVED WITH IMAGE = stats - 3"
setimhead l,"stats-3"
clall
wait 100000
endfor
stop

#####################################
write "     THIS IS THE END OF THE MACRO "
write "     CALLED  ***  1MO-AD.MCR  ***  "
#####################################


##########################################
#                          #
# MACRO                    # SEGMENTATION
#                          #
# TITLE                    # IMAGE SEGMENTATION
#                          #
# DESCRIPTION              #LARGE SCALE SEGMENTATION OF
#                          # MANY IMAGES
#                          #
# DATE  WRITTEN            # 19/02/93
#                          #
# SAVED AS                 # 1MO-Seg.(mcr)
#                          #
##########################################

macro setup
endmacro

###  LIST OF MACROS OPERATIONAL  ###

setup   # FULL INITIALIZATION
imar    # RETRIEVAL
seg     # SEGMENTATION
corev   # CONTOUR AND REVIEW
stop

# SEGMENTATION - 6 IMAGES WITH CHECK AND CORRECTION

write " SEGMENTATION function"
display 1
write "                    dis2lev 1,2,0,160,_ON,_ON,1"
dis2lev 1,2,0,160,_ON,_ON,1
display 2
write "                    contour 2,1,3,7,128,_ON,_ON"
contour 2,1,3,7,128,_ON,_ON
write "                    outlink 3,3,3,9,3,_ON"
outlink 3,3,3,9,255,_OFF
write "                    dis2lev 3,3,255,255,_ON,_ON,1"
dis2lev 3,3,255,255,_ON,_OFF,1
clovl 1
display 1
write "                    combine 2,3,1,0,0.5,0.5"
combine 2,3,1,0,0.5,0.5
clearmes
display 4
write "                    dis2lev 4,5,0,160,_ON,_ON,1"
dis2lev 4,5,0,160,_ON,_ON,1
display 5
```

```
write "                    contour 5,4,6,7,128,_ON,_ON"
contour 5,4,6,7,128,_ON,_ON
write "                    outlink 6,6,6,1,0,_ON"
outlink 6,6,6,9,255,_OFF
write "                    dis2lev 6,6,255,255,_ON,_ON,1"
dis2lev 6,6,255,255,_ON,_OFF,1
clovl 4
display 4
write "                    combine 5,6,4,0,0.5,0.5"
combine 5,6,4,0,0.5,0.5
write "The End of Segmentation, Now For Storage"


# SEGMENTATION - 3 IMAGES

write " SEGMENTATION function"
write "  3  -  ....at 2 threshold levels;    "
write "IMAGE NUMBER    :  1  ",m
display 1
dis2lev 1,4,0,160,_ON,_ON,1
write "IMAGE NUMBER    :  1  ",n
display 4
dis2lev 2,5,0,160,_ON,_ON,1
write "IMAGE NUMBER    :  1  ",o
display 5
dis2lev 3,6,0,160,_ON,_ON,1
write "The end of segmentation, now for storage"
display 6


# SEGMENTATION - 3 IMAGES WITH INTENSIVE CORRECTION

write " SEGMENTATION function"
write "  3  -  ....at 2 Threshold Levels,    "
write "IMAGE NUMBER    :  1  ",m
display 1
dis2lev 1,2,0,160,_ON,_ON,1
write "IMAGE NUMBER    :  1  ",n
display 4
dis2lev 4,5,0,160,_ON,_ON,1
write "The End of Segmentation, now for EDITING"

display 1
scrap 1,2,_OFF,0,100,_ON,_ON
identify 2,2,_ON,_OFF
outlink 2,2,6,3,212,_ON
fill 2,2
identify 2,3,_ON,_OFF
rejectobj 2,3,0
identframe 1,2,_ON,1,256,256,0,0,_ON,_ON,_ON
outlink 2,3,6,3,214,_ON
scrap 3,3,_OFF,0,100,_ON,_ON

display 4
scrap 5,5,_OFF,0,100,_ON,_ON
identify 5,5,_ON,_OFF
outlink 5,5,6,1,0,_ON
identify 5,6,_ON,_OFF
fill 5,5
rejectobj 5,5,0
identframe 5,6,_ON,1,167,54,327,338,_ON,_ON,_ON
loadlut "grey"


# AUTOMATIC RETRIEVAL, MANUAL SEGMENTATION AND AUTOMATIC STORAGE

call
for i=11,1<100,i=i+5
getim "sta5-on",1
beep 500,300
dis2lev 1,2,64,191,_ON,_ON,1
dis2lev 1,3,64,191,_ON,_ON,1
dis2lev 1,4,64,191,_ON,_ON,1
dis2lev 1,5,64,191,_ON,_ON,1
dis2lev 1,6,64,191,_ON,_ON,1
write "end of discrimination"
storim i,2
write i
storim i+1,3
write i
storim i+2,4
write i
storim i+3,5
write i
storim i+4,6
write "the end of storing images "
endfor

##################################
write "      THIS IS THE END OF THE MACRO "
write "      CALLED     ***    1MO-seg.MCR     ***     "
write "            IMAGE SEGMENTATION FUNCTIONS  "
##########################################


##################################
#                                #
# MACRO                          # MEASUREMENT VERSION ONE
#                                #
# TITLE                          # BINARY IMAGE MEASUREMENT
#                                #
# DESCRIPTION                    # AUTOMATIC MEASUREMENT OF
#                                # SEGMENTED IMAGES
#                                #
# DATE  WRITTEN                  # 07/12/92
#                                #
# SAVED AS                       # 1MO-MEAS_1 (mor)
#                                #
##########################################

macro setup
endmacro

### LIST OF MACROS OPERATIONAL ###

setup               # FULL INITIALIZATION
pset                # PARTIAL INITIALIZATION
scale               # SCALE FUNCTIONS
medbp               # USER MEAS & DB PARAMETERS
dbinf               # SET DB INFO
imar                # RETRIEVAL
smdbp               # OPENING DB AND MEASURING
res                 # RESULTS
corev               # CONTOUR AND REVIEW
stop


setup
medbp
alpha
setimpath "f:/bld/comu-30"
mogrtxt2 = "IGS-"
write mogrtxt2
for i=1, i<70, i=i+1
pset
clearmes
n=1
getim mogrtxt2 + string(i),n
write "IMAGE     ",mogrtxt2 + string(i),"  RETRIEVED TO :  ",n
write "IMAGE     ",mogrtxt2 + string(i+1),"  RETRIEVED TO :  ",n+1
getim mogrtxt2 + string(i+1),n+1
getim mogrtxt2 + string(i+2),n+2
write " IMAGE     ",mogrtxt2 + string(i+2),"  RETRIEVED TO   :",n+2
write " IMAGE     ",mogrtxt2 + string(i+3),"  RETRIEVED TO   :",n+3
getim mogrtxt2 + string(i+4),n+4
getim mogrtxt2 + string(i+5),n+5
write "NOW RETRIEVED SIX CONSECUTIVE IMAGES ",mogrtxt2 + string(i),"
to ",i+6
wait 100
scale
wait 100
smdbp
DBopen "co","mgfeat1"
DBopen "co-2","mgfeat6"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "co"
if (_STATUS)  break
DBappend "co-2"
endwhile
DBclose "co"
DBclose "co-2"
DBopen "ro","mgfeat2"
DBopen "ro-2","mgfeat7"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "ro"
if (_STATUS) : break
DBappend "ro-2"
endwhile
DBclose "ro"
DBclose "ro-2"
DBopen "fs","mgfeat3"
DBopen "fs-2","mgfeat8"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "fs"
if (_STATUS) : break
DBappend "fs-2"
endwhile

DBclose "fs"
DBclose "fs-2"
DBopen "fh","mgfeat4"
DBopen "fh-2","mgfeat9"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "fh"
if (_STATUS) : break
DBappend "fh-2"
endwhile

DBopen "all","mgfeat5"
DBopen "all-2","mgfeat10"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "all"
if (_STATUS)  break
DBappend "all-2"
endwhile

DBclose "co"
DBclose "ro"
DBclose "fs"
DBclose "fh"
DBclose "all"
wait 100


# OBJECTSPECIFIC PARAMETERS

InitObj AREA,DMAX,DMIN,FCIRCLE,ACPX,ACPY
InitObj AREA,DMAX,DMIN,FCIRCLE,CGRAVY,CGRAVX
AREA = DMAX + DMIN + FCIRCLE + CGRAVY + CGRAVX + ACPX + ACPY
+ 0
global ELONG
ELONG = 0.0
mgfeat1[]=AREA,FCIRCLE,ELONG
mgfeat2[]=AREA,FCIRCLE,ELONG
mgfeat3[]=AREA,FCIRCLE,ELONG
mgfeat4[]=AREA,FCIRCLE,ELONG
mgfeat5[]=AREA,FCIRCLE,ELONG

DBerase "co"
DBcreate "co","mgfeat1"
DBclose "co"
DBerase "ro"
DBcreate "ro","mgfeat2"
DBclose "ro"
DBerase "fs"
DBcreate "fs","mgfeat3"
DBclose "fs"
DBerase "fh"
DBcreate "fh","mgfeat4"
DBclose "fh"
DBerase "all"
DBcreate "all"
DBclose "all"
wait 1000
endfor
stop

outlist "ro",_OFF
outlist "ro-2",_OFF
outlist "co",_OFF
outlist "co-2",_OFF
outlist "fh",_OFF
outlist "fh-2",_OFF
outlist "fs",_OFF
outlist "fs-2",_OFF
outlist "all",_OFF
outlist "all-2",_OFF
outlist "rs",_OFF
outlist "mgfield",_OFF

outhist"ro-2","AREA",15,_OFF,_OFF,0,_ON,_OFF,0.00,0.1,450,"","HISTOGR
AM"
outhist"ro-2","ELONG",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","HI
STOGRAM"
outhist"ro-2","FCIRCLE",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","
HISTOGRAM"
outhist"ro-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","
HISTOGRAM"
outhist"co-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","
HISTOGRAM"
outhist"co-2","FCIRCLE",15,_OFF,_OFF,0,_ON,_ON,0.00,0.1,100.00,"","HIS
TOGRAM"
outhist"co-2","ELONG",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","HI
STOGRAM"
outhist"co-2","AREA",15,_OFF,_OFF,0,_ON,_OFF,0.00,0.06,200,"","HISTOG
RAM"
outhist"co-2","AREA",15,_OFF,_OFF,0,_ON,_OFF,0.00,0.06,200,"","HISTOG
RAM"
outhist"mgfield","FIELDCOUNT",15,_OFF,_OFF,0,_ON,_ON,0.00,0.06,200.0
0,"","HISTOGRAM"
outhist"all-2","AREA",15,_OFF,_OFF,0,_ON,_ON,0.00,0.06,200.00,"","HISTO
GRAM"
outhist"fs-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,1.00,108.10,59.00,"","HI
STOGRAM"
outhist"fh-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,1.00,108.10,59.00,"","HI
STOGRAM"

##########################################
write "      THIS IS THE END OF THE MACRO "
write "      CALLED     ***    1MO-MEAS_1.MCR     ***     "
##########################################


##################################
#                                #
# MACRO                          # MEASUREMENT VERSION TWO
#                                #
# TITLE                          # USER SELECTED OBJECT
#                                # MEASUREMENT
#                                #
# DESCRIPTION                    # USED TO SELECTIVELY MEASURE
#                                #  MICROORGANISMS IN
SEGMENTED IMAGES
#                                #
# SAVED AS                       # 1MO-MEA_2 (mor)
#                                #
##########################################

macro setup
endmacro

### LIST OF MACROS OPERATIONAL ###

setup                  # FULL INITIALIZATION
pset                   # PARTIAL INITIALIZATION
scale_3                # SCALE FUNCTIONS
medbp_3                # USER MEAS & DB PARAMETERS
dbinf                  # DATA BASE INFORMATION
imar_3                 # RETRIEVAL
seg_2                  # SEGMENTATION
smdbp                  # OPENING DB AND MEASURING
resul                  # RESULTS
corev                  # CONTOUR AND REVIEW
stop
setimpath "f:/bld/mo-gr1/images/calibrat"


# MEASUREMENT AND DATA BASE PARAMETERS

macro smdbp
for n=1,n<7,n=n+3
clearmes
loadlut "ident"
display n
text1="co"
text2="ro"
```

```
text3="fs"
text4="fh"
text5="all"
text21="mgfield"
text11="mgfeat1"
text12="mgfeat2"
text13="mgfeat3"
text14="mgfeat4"
text15="mgfeat5"
write "USING IMAGE No.   ",n," FOR MEASUREMENT"

# FIELDBASED PARAMETERS

DBopen text21,"mgfield"
Measf n
DBappend text21
write "No. of bacteria in image     ",n," * ",FIELDCOUNT
DBclose text21

# OBJECTSPECIFIC PARAMETERS

for i=1, i<2, i=i+1
name1=text1    # + string(i)
name2=text11   # + string(i)
DBopen name1,name2
endfor

for i=1, i<2, i=i+1
name1=text2   # + string(i)
name2=text12  # + string(i)
DBopen name1,name2
endfor

for i=1, i<2, i=i+1
name1=text3   # + string(i)
name2=text13  # + string(i)
DBopen name1,name2
endfor

for i=1, i<2, i=i+1
name1=text4   # + string(i)
name2=text14  # + string(i)
DBopen name1,name2
endfor

for i=1, i<2, i=i+1
name1=text5
name2=text15
DBopen name1,name2
endfor

Gstring n,10,440,"1    = CO",-1,0,0
Gredg n,10,440,10,10,13,0
Gstring n,20,450,"2    = RO",-1,0,0
Gredg n,20,450,10,10,5,0
Gstring n,30,460,"3    = FS",-1,0,0
Gredg n,30,460,10,10,15,0
Gstring n,40,470,"4    = FH",-1,0,0
Gredg n,40,470,10,10,9,0
Gstring n,50,480,"5    = OTHER",-1,0,0
Gredg n,50,480,10,10,11,0
Gstring n,90,490,"AREA (COLOUR); ELONGATION THEN FCIRCLE",-1,0,0
write " HIT <ESC> TO STOP COUNTING"
write " What select object to measure (white), then classify!! "

display n
while (1)
   write " Hit <ESC> to stop counting"
   Goursori n,x,y,1
   if _STATUS eq 27: break
   Measoi n,x,y
   ELONG=DMAX/DMIN
write " What type does this object belong to  (1/2/3/4/5)   : "
    Goursori n,x,y,1
  if (x >=10 && x <=20 && y >=440 && y <=450) : DBwrite text1
  endif
  if (x >=20 && x <=30 && y >=450 && y <=460) : DBwrite text2
  endif
  if (x >=30 && x <=40 && y >=460 && y <=470) : DBwrite text3
  endif
  if (x >=40 && x <=50 && y >=470 && y <=480) : DBwrite text4
  endif
  if (x >=50 && x <=60 && y >=480 && y <=490) : DBwrite text5
  endif

x=int(ACPX)
y=int(ACPY)
Gstring n,x,y,int(AREA*100),-1,1,0
Gstring n,x,y+25,int(ELONG*100),-1,1,0
Gstring n,x,y+50,int(FCIRCLE*100),-1,1,0
endwhile

DBclose text1
DBclose text2
DBclose text3
DBclose text4
DBclose text5
write "..............the measurement is completed."
endfor
endmacro

DBopen "co","mgfeat1"
DBopen "co-2","mgfeat6"
AREA=FCIRCLE=ELONG=0
USERNO=1
while (1)
DBread "co"
if (_STATUS) : break
DBappend "co-2"
endwhile
DBclose "co"
DBclose "co-2"

DBopen "ro","mgfeat2"
DBopen "ro-2","mgfeat7"
AREA=FCIRCLE=ELONG=0
USERNO=1
while (1)
DBread "ro"
if (_STATUS) : break
DBappend "ro-2"
endwhile
DBclose "ro"
DBclose "ro-2"

DBopen "fs","mgfeat3"
DBopen "fs-2","mgfeat8"
AREA=FCIRCLE=ELONG=0
USERNO=1
while (1)
DBread "fs"
if (_STATUS) : break
DBappend "fs-2"
endwhile
DBclose "fs"
DBclose "fs-2"

DBopen "fh","mgfeat4"
DBopen "fh-2","mgfeat9"
AREA=FCIRCLE=ELONG=0
USERNO=1
while (1)
DBread "fh"
if (_STATUS) : break
DBappend "fh-2"
endwhile

DBopen "all","mgfeat5"
DBopen "all-2","mgfeat10"
AREA=FCIRCLE=ELONG=0
USERNO=1
while (1)
DBread "all"
if (_STATUS) : break
DBappend "all-2"
endwhile

DBclose "co"
DBclose "ro"
DBclose "fs"
DBclose "fh"
DBclose "all"
wait 100

OBJECTSPECIFIC PARAMETERS
IntObj AREA,DMAX,DMIN,FCIRCLE,ACPX,ACPY
IntObj AREA,DMAX,DMIN,FCIRCLE,CGRAVY,CGRAVX
AREA = DMAX = DMIN = FCIRCLE = CGRAVY = CGRAVX = ACPX = ACPY
= 0
global ELONG
ELONG = 0.0
mgfeat1[]=AREA,FCIRCLE,ELONG
mgfeat2[]=AREA,FCIRCLE,ELONG
mgfeat3[]=AREA,FCIRCLE,ELONG
mgfeat4[]=AREA,FCIRCLE,ELONG
mgfeat5[]=AREA,FCIRCLE,ELONG

DBerase "co"
DBcreate "co","mgfeat1"
DBclose "co"
DBerase "ro"
DBcreate "ro","mgfeat2"
DBclose "ro"
DBerase "fs"
DBcreate "fs","mgfeat3"
DBclose "fs"
DBerase "fh"
DBcreate "fh","mgfeat4"
DBclose "fh"
DBerase "all"
DBcreate "all"
DBclose "all"
wait 1000
endfor
stop

#####################################
write "   THIS IS THE END OF THE MACRO "
write "   CALLED    ***   1MO-MEAS_2.MCR   *** "
#####################################


#########################################
#                                       #
# MACRO                                 # MEASUREMENT VERSION 3
#                                       #
# TITLE                                 # MEASUREMENT OF MULTIPLE
#                                       # IMAGES
#                                       #
# DESCRIPTION                           # MEASUREMENT OF LARGE
#                                       # NUMBERS OF
#                                       # SEGMENTED IMAGES.
#                                       #
# DATE  WRITTEN                         # 10/09/93
#                                       #
#                                       #
# SAVED AS                              # 1MO-MEA3 (mcr)
#                                       #
#########################################

macro setup
endmacro

###  LIST OF MACROS OPERATIONAL   ###

setup                   # FULL INITIALIZATION
pset                    # PARTIAL INITIALIZATION
scale                   # SCALE FUNCTIONS
medbp                   # USER MEAS & DB PARAMETERS
dbinf                   # SET DB INFO FIRST
imar                    # RETRIEVAL
smdbp                   # OPENING DB AND MEASURING
res                     # RESULTS
corev                   # CONTOUR AND REVIEW
setup
medbp
res
setimpath "f:/bld/ccmu-23"
mogrtxt2 = "C23-"
write mogrtxt2

for i=15, i<28, i=i+1
pset
clearmes
n=1
getim mogrtxt2 + string(i),n
write "IMAGE    :  ",mogrtxt2 + string(i)," RETRIEVED TO :  ",n
write "ref IMAGE : ref   RETRIEVED TO :2 ",n+1
getim mogrtxt2 + string(i-14),n+1
write " IMAGE    :  ",mogrtxt2 + string(i+2)," RETRIEVED TO   ",n+2
write " IMAGE    :  ",mogrtxt2 + string(i+3)," RETRIEVED TO   ",n+3
getim mogrtxt2 + string(i+4),n+4
getim mogrtxt2 + string(i+5),n+5
wait 100
scale
wait 100
smdbp

#PRINTING OF RESULTS ON SCREEN TO PRINTER
plotim  n+1,3,_OFF,1,1,15,15,
_OFF,_OFF,_ON,_OFF,"plot.hlt"
plotim n+1,3,_OFF,1,1,15,15,
_OFF,_ON,_ON,_OFF,"plot.hlt"
res

#####################################################
write "   THIS IS THE END OF THE MACRO "
write "   CALLED    ***   1MO-meas_3.MCR   ***   "
#####################################


#########################################
#                                       #
# MACRO                                 # REPRODUCIBILITY
#                                       #
# TITLE                                 # TEST OF REPRODUCIBILITY
#                                       #
# DESCRIPTION                           # USED TO CHECK
#                                       # REPRODUCIBILITY OF IA
#                                       #
# DATE  WRITTEN                         # 12/04/93
#                                       #
# SAVED AS                              # 1MO-ST-1 (mcr)
#
#########################################

macro setup
endmacro

###  LIST OF MACROS OPERATIONAL   ###

setup           # FULL INITIALIZATION
pset            # PARTIAL INITIALIZATION
scale           # SCALE FUNCTIONS
medbp           # USER MEAS & DB PARAMETERS
dbinf           # SET DB INFO FIRST
imar            # RETRIEVAL
bgcor           # BACKGROUND CORRECTION
filtr           # FILTER ENHANCEMENT
seg             # SEGMENTATION
binp            # BINARY IMAGE PROCESSING
ident           # IDENTIFICATION
smdbp           # OPENING DB AND MEASURING
resul           # RESULTS
corev           # CONTOUR AND REVIEW
stop

for m=18, m<101, m=m+1
write "You have NOW received image number  :  ",m
clall
loadlut "grey"
getim m,5
wait 10000
btdscale
wait 10000
btdbgcor
btdfiltr
btdseg
btdbinp
wait 10000
btdident
btdsmdbp
wait 10000
DBopen "MOGR-DB2","mgfeat2"
write "No. of bactera in this image =  ",FIELDCOUNT
wait 10000
endfor
stop
outlist "MOGR-DB2"._OFF
outlist "MOGR-DB3"._OFF

#####################################
write "   THIS IS THE END OF THE MACRO "
write "   CALLED    ***   1MO-el-1 MCR   ***   "
#####################################
```

APPENDIX FIVE : IMAGE ANALYSIS DEMONSTRATION MACROS

```
#########################################
#                                       #
# MACRO                 # DEMONSTRATION MACRO ONE
#                       #
# TITLE                 # DEMONSTRATION OF BASIC
#                       # PROCEDURE TO
#                       # ANALYZE AN IMAGE
#                       #
# DESCRIPTION           # THIS MACRO USES STORED
#                       # IMAGEs TO
#                       # DEMONSTRATE HOW AREA
#                       # DETERMINATIONS
#                       # ARE ACHIEVED
#                       #
# DATE  WRITTEN         # 19/01/93
#                       #
# SAVED AS              # 1MO-demo (mcr)
#                       #
#########################################


# START HERE - PRESS F2 AND THEN F9
for i=1,i<10,i=i+1


###  FULL INITIALIZATION  ###

write "INITIALIZATION"
clearall
clearallov
clall
clearmes
measalop
resetpar
resetlim
alpha
setimpath "F:/images"
setframe "FS12"
loadlut "grey"
ovcolour 3
clear 1,0
display 1
tvchan 0,0


###  SCALE FUNCTIONS  ###

write ""
write " Use an already calibrated scale (2)"
display 1
scalgeom 1,"mag=400x",_OFF,_OFF
marker 1,10,10,_ON,_ON,_ON,2


# IMAGE RETRIEVAL

write "IMAGE RETRIEVAL"
getim "ct-on",1
write ""
write "ORIGINAL IMAGE"
wait 5000
write ""
write "THIS IMAGE IS NORMALIZED....."
getim "ct-norm",2
write ""
write "THE IMAGE BACKGROUND IS CORRECT TO COUNTER DIFFERENT
LIGHT INTENSITIES"
wait 5000
getim "ct-bgcor",3
wait 5000
write ""
write "THE BACTERIA EDGES ARE ENHANCED WITH A FILTER ....."
getim "ct-filt",4
write ""
write "SEGMENTATION OCCURS ....."
wait 5000
getim "ct-seg",5
write ""
write "AS BACTERIA ARE LARGER THAN 30 PIXELS, ANYTHING UNDER
THIS IS REMOVED"
wait 5000
getim "ct-scrap",6
loadlut "dent"
write ""
write "THE BACTERIA ARE IDENTIFIED FOR MEASUREMENT ....."
wait 5000
getim "ct-idfra",7
InitObj AREA,CGRAVX,CGRAVY
write ""
write "THE BACTERIA ARE NOW MEASURED....."
wait 5000
measobj 7,"CT-94-AD"._OFF
scrap 7,7,_OFF,0,0,_ON,_ON
loadlut "grey"
write ""
write "TO ENSURE THAT THE MEASUREMENT IS TRUE, THE MEASURED
BACTERIA ARE OVERLAID"
contour 7,3,8,7,128,_ON,_OFF
wait 10000
copy 3,9
DBopen "CT-94-AD","mo-feat1"
write ""
write "THE RESULTS ARE WRITTEN TO THE SCREEN"
wait 5000
while 1
DBread "CT-94-AD"
if _STATUS.break
Gwring 9,int(CGRAVX*5),
int(CGRAVY*5+15),int(AREA),-1,1,0
wait 500
endwhile
DBclose "CT-94-AD"
write ""
write "THE END OF MACRO ####     1MO-DEMO      ####### "

#########################################
write "       THIS IS THE END OF THE MACRO "
write "      CALLED     ***     1MO-demo.MCR     *** "
#########################################
endfor
```

```
#########################################
#                                       #
# MACRO                 # DEMONSTRATION MACRO TWO
#                       #
# TITLE                 # ANALYSIS OF LARGE
#                       # NUMBERS OF IMAGES
#                       #
# DESCRIPTION           # DEMONSTRATION OF BASIC PROCEDURE TO
#                       # ANALYZE A LARGE NUMBER OF IMAGES
#                       #
# DATE                  # 03/02/93
#                       #
# SAVED AS              # 1MO-demo2.(mcr)
#                       #
#########################################


macro setup
endmacro


###  LIST OF MACROS REQUIRED  ###


setup           # FULL INITIALIZATION
pset            # PARTIAL INITIALIZATION
scale           # SCALE FUNCTIONS
medbp           # USER MEAS & DB PARAMETERS
dbint           # SAT DB INFO FIRST
imar            # RETRIEVAL
smdbp           # OPENING DB AND MEASURING
resul           # RESULTS
corev           # CONTOUR AND REVIEW
stop


#start here


for m=1,m<100,m=m+1
text1="bf-60b-"
for i=1,i<4,i=i+1
bldpset
wait 1000
name1=text1 + string(i)
getim name1,1
write "IMAGE NUMBER    ",name1," RETRIEVED TO :   1"
copy 1,2
wait 1000
scale
wait 1000
smdbp

DBopen "co","mgfeat1"
DBopen "co-2","mgfeat6"
AREA=FCIRCLE+ELONG+0
USERNO=i
while (1)
DBread "co"
if (_STATUS) : break
DBappend "co-2"
endwhile
DBclose "co"
DBclose "co-2"

DBopen "ro","mgfeat2"
DBopen "ro-2","mgfeat7"
AREA=FCIRCLE+ELONG+0
USERNO=i
while (1)
DBread "ro"
if (_STATUS) : break
DBappend "ro-2"
endwhile
DBclose "ro"
DBclose "ro-2"

DBopen "fs","mgfeat3"
DBopen "fs-2","mgfeat8"
AREA=FCIRCLE+ELONG+0
USERNO=i
while (1)
DBread "fs"
if (_STATUS) : break
DBappend "fs-2"
endwhile
DBclose "fs"
DBclose "fs-2"

DBopen "th","mgfeat4"
DBopen "th-2","mgfeat9"
AREA=FCIRCLE+ELONG+0
USERNO=i
while (1)
DBread "th"
if (_STATUS) : break
DBappend "th-2"
endwhile

DBopen "all","mgfeat5"
DBopen "all-2","mgfeat10"
AREA=FCIRCLE+ELONG+0
USERNO=i
while (1)
DBread "all"
if (_STATUS) : break
```

```
DBappend "all-2"
endwhile

DBclose "co"
DBclose "ro"
DBclose "fs"
DBclose "fh"
DBclose "all"
wait 100


# OBJECTSPECIFIC PARAMETERS

InitObj AREA,DMAX,DMIN,FCIRCLE,ACPX,ACPY
AREA = DMAX = DMIN = FCIRCLE = CGRAVY = CGRAVX = ACPX = ACPY
= 0
global ELONG
ELONG = 0.0
mgfeat1[]=AREA,FCIRCLE,ELONG
mgfeat2[]=AREA,FCIRCLE,ELONG
mgfeat3[]=AREA,FCIRCLE,ELONG
mgfeat4[]=AREA,FCIRCLE,ELONG
mgfeat5[]=AREA,FCIRCLE,ELONG

DBerase "co"
DBcreate "co","mgfeat1"
DBclose "co"
DBerase "ro"
DBcreate "ro","mgfeat2"
DBclose "ro"
DBerase "fs"
DBcreate "fs","mgfeat3"
DBclose "fs"
DBerase "fh"
DBcreate "fh","mgfeat4"
DBclose "fh"
DBerase "all"
DBcreate "all"
DBclose "all"
wait 1000
endfor
endfor
endfor
stop


# RESULTS

outlist "ro",_OFF
outlist "ro-2",_OFF
utlist "co-2",_OFF
outlist "fh",_OFF
outlist "fh-2",_OFF
outlist "fs",_OFF
outlist "fs-2",_OFF
outlist "mgfield",_OFF

outhist"ro-2","AREA",15,_OFF,_OFF,0,_ON,_OFF,0,0,0,"","HISTOGRAM"
outhist"ro-2","ELONG",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"ro-2","FCIRCLE",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"ro-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"co-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"co-2","FCIRCLE",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"co-2","ELONG",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"co-2","AREA",15,_OFF,_OFF,0,_ON,_OFF,0,0,0,"","HISTOGRAM"
outhist"co-2","AREA",15,_OFF,_OFF,0,_ON,_OFF,0,0,0,"","HISTOGRAM"
outhist"mgfield","FIELDCOUNT",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTO
GRAM"
outhist"all-2","AREA",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"fs-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
outhist"fh-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"

##############################
write "      THIS IS THE END OF THE MACRO "
write "      CALLED     ***    1MO-DEMO2.MCR     ***    "
##########################################
```

```
##########################################
#                                        #
# MACRO                  # DEMONSTRATION MACRO THREE
#                                        #
# TITLE                  # ANALYSIS OF IMAGES OF
#                        # DIFFERENT
#                        # MAGNIFICATION
#                                        #
# DESCRIPTION            # THIS MACRO IS USED TO
#                        # ANALYSE
#                        # MULTIPLE IMAGES WITH
#                        # DIFFERENT
#                        # MAGNIFICATION
#                                        #
# DATE  WRITTEN          # 19/06/94
#                                        #
# SAVED AS               # 1MO-DEMO3.(mcr)
#                                        #
##########################################


macro setup
endmacro


###  LIST OF MACROS OPERATIONAL   ###


setup          # FULL INITIALIZATION
pset           # PARTIAL INITIALIZATION
scale          # SCALE FUNCTIONS
medbp          # USER MEAS & DB PARAMETERS
dbinf          # SET DB INFO FIRST
imar           # RETRIEVAL
smdbp          # OPENING DB AND MEASURING
resul          # RESULTS
corev          # CONTOUR AND REVIEW


for m=1,m<100,m=m+1

text1="bfde3a"
text2="bfde3b"

for i=1,i<4,i=i+1
pset
wait 1000
name1=text1 + string(i)
getim name1,1
write "IMAGE        ",name1,"  RETRIEVED TO :   1"

write " Use an already calibrated scale (2)"
wait 1000
write "IMAGE   :   ",name1,"      IS NOW OVERLAID WITH SCALE  "
if i == 1
scalgeom 1,"CZ6BZ",_OFF,_OFF    # 0.1
endif
if i == 2
scalgeom 1,"CO3B5",_OFF,_OFF    # 0.005
endif
if i == 3
scalgeom 1,"CZ6B4",_OFF,_OFF    # 0.02
endif
wait 1000
marker 1,1,1,_ON,_ON,_ON,2
namebin=text2 + string(i)
wait 1000
write namebin
getim namebin,2

smdbp

dis2lev 3,3,1,255,_ON,_OFF,1
loadlut "grey"
contour 3,1,4,7,128,_ON,_ON
display 4

DBopen "co","mgfeat1"
DBopen "co-2","mgfeat6"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "co"
if (_STATUS) : break
DBappend "co-2"
endwhile
DBclose "co"
DBclose "co-2"

DBopen "ro","mgfeat2"
DBopen "ro-2","mgfeat7"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "ro"
if (_STATUS) : break
DBappend "ro-2"
endwhile
DBclose "ro"
DBclose "ro-2"

DBopen "fs","mgfeat3"
DBopen "fs-2","mgfeat8"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "fs"
if (_STATUS)  break
DBappend "fs-2"
endwhile
DBclose "fs"
DBclose "fs-2"
```

```
DBopen "fh","mgfeat4"
DBopen "fh-2","mgfeat9"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "fh"
if (_STATUS) : break
DBappend "fh-2"
endwhile

DBopen "all","mgfeat5"
DBopen "all-2","mgfeat10"
AREA=FCIRCLE=ELONG=0
USERNO=i
while (1)
DBread "all"
if (_STATUS) : break
DBappend "all-2"
endwhile

DBclose "co"
DBclose "ro"
DBclose "fs"
DBclose "fh"
DBclose "all"
wait 100


# OBJECTSPECIFIC PARAMETERS

InitObj AREA,DMAX,DMIN,FCIRCLE,ACPX,ACPY
AREA = DMAX = DMIN = FCIRCLE = CGRAVY = CGRAVX = ACPX = ACPY
= 0
global ELONG
ELONG = 0.0
mgfeat1[]=AREA,FCIRCLE,ELONG
mgfeat2[]=AREA,FCIRCLE,ELONG
mgfeat3[]=AREA,FCIRCLE,ELONG
mgfeat4[]=AREA,FCIRCLE,ELONG
mgfeat5[]=AREA,FCIRCLE,ELONG
DBerase "co"
DBcreate "co","mgfeat1"
DBclose "co"
DBerase "ro"
DBcreate "ro","mgfeat2"
DBclose "ro"
DBerase "fs"
DBcreate "fs","mgfeat3"
DBclose "fs"
DBerase "fh"
DBcreate "fh","mgfeat4"
DBclose "fh"
DBerase "all"
DBcreate "all"
DBclose "all"
wait 1000
outhist"all-2","AREA",15,_OFF,_OFF,0,_ON,_ON,0,0,0,"","HISTOGRAM"
endfor
endfor
endfor
stop


# RESULTS

outlist "ro",_OFF
outlist "ro-2",_OFF
outhist"ro-2","AREA",15,_OFF,_OFF,0,_ON,_ON,0.00,0.1,450,"","HISTOGRAM"
outhist"ro-2","ELONG",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","HI
STOGRAM"
outhist"ro-2","FCIRCLE",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","
HISTOGRAM"
outhist"ro-2","USERNO",15,_OFF,_OFF,0,_ON,_ON,0.00,100.00,100.00,"","
HISTOGRAM"
outhist

##################################
write "      THIS IS THE END OF THE MACRO "
write "      CALLED     ***    1MO-DEMO3.MCR     ***    "
######################################
```

Before grey-level values could be used a standard light intensity curve had to be constructed. This was done by capturing images of an uninoculated CCMU at 400X microscope magnification from a Zeiss Axiophot microscope. The area analyzed.field^{-1} was 4920μm^2 (768 X 512 pixels). Each image was obtained at an increased light intensity by controlling the amount of light in the microscope from dark (1) to full light (9). The values GREYM, GREYSD, TRANSMM, TRANSMSD, OPTDM, OPTDSD and OPTDI were measured for each image (Kontron Elektronik, 1991) where GREYM = Mean grey value inside object; GREYSD = Standard deviation of grey value; TRANSMM = Mean transmission of object (%); TRANSMSD = Standard deviation of the transmission; OPTDM = Mean optical density; OPTDSD = Standard deviation of the optical density; and OPTDI = Integrated optical density (OPTDI = OPTDM X AREA).

The standard deviations of the grey value and the transmission (GREYSD and TRANSMSD) from the standard curve of increased light intensities are shown (Figure A.6.1 and Table A.6.1). The area value, as expected with field based measurements, remained constant (4920μm^2) for each image. The transmission and grey-level values increased while the optical density decreased for the standard curve of increased light intensity.

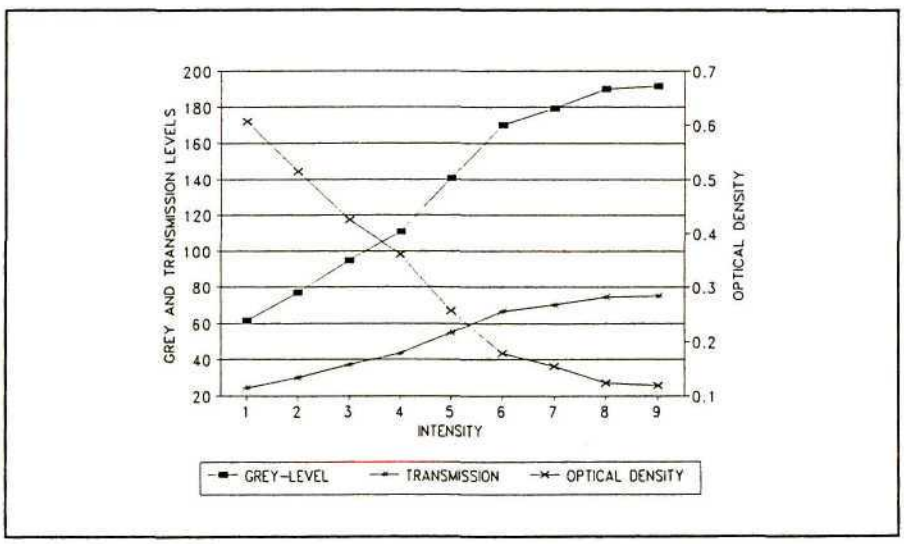

Figure A.6.1 Standard curve of grey-level, transmission and optical density at different light intensities. A Zeiss Axiophot microscope was used to create the images. A Kontron Vidas 2.1 Image Analyzer produced the results.

Table A.6.1. Standard deviations of the grey-level and transmission values at different light intensities. A Zeiss Axiophot microscope was used to create the images. A Kontron Vidas 2.1 Image Analyzer produced the results.

IMAGE NUMBER (INCREASING LIGHT)	STANDARD DEVIATION OF GREY-LEVEL (GREYSD)	STANDARD DEVIATION OF TRANSMISSION (TRANSMSD)	STANDARD DEVIATION OF OPTICAL DENSITY (OPTDSD)
1	1.5562	0.610	0.044
2	5.6478	2.215	0.064
3	10.2427	4.018	0.046
4	13.7693	5.399	0.046
5	19.6897	7.722	0.050
6	25.1234	9.852	0.052
7	145.8606	57.20	0.041
8	146.8873	57.60	0.043
9	147.1454	57.70	0.049