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# MICROBIOLOGICAL QUALITY ASSURANCE

## A Guide Towards Relevance *and* Reproducibility *of* Inocula

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# 3.8 Screening for Novel Compounds/Activity in the Environmental Protection Industries

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## INTRODUCTION

Bacteria have many useful properties that may be harnessed to industrial processes. These include the production of antibiotics, enzymes for effecting biotransformations, and a variety of pharmaceutical compounds, together with the use of microorganisms as vehicles for the synthesis of bioengineered products. In such processes the growth of these bacteria is well controlled and optimized for the process in hand. It is not necessary under such circumstances to duplicate *in vivo/in situ*. In many industrial processes, however, and sometimes within manufacturing plants, microorganisms can grow in an uncontrolled manner as contaminants or nuisances. In such circumstances they often grow attached to surfaces, fouling them with their growth products and eventually destroying the materials' surface, either through obstruction of the flow of materials over them or through biocorrosion. The control of biofouling/biocorrosion microorganisms forms the basis of this chapter. Methods for their control have, to date, focused on tests which deploy antimicrobial agents against pure cultures of vegetatively growing planktonic bacteria. This chapter highlights the disadvantages and inappropriateness of such methods and offers suggestions for the design of more relevant laboratory testing procedures.

## THE INDUSTRIAL PROCESSES

In all of the natural environments studied to date, including rivers, lakes, soils, and estuaries, bacteria have been shown to attach to surfaces and grow within biofilms as sessile populations of cells. In the same manner bacteria

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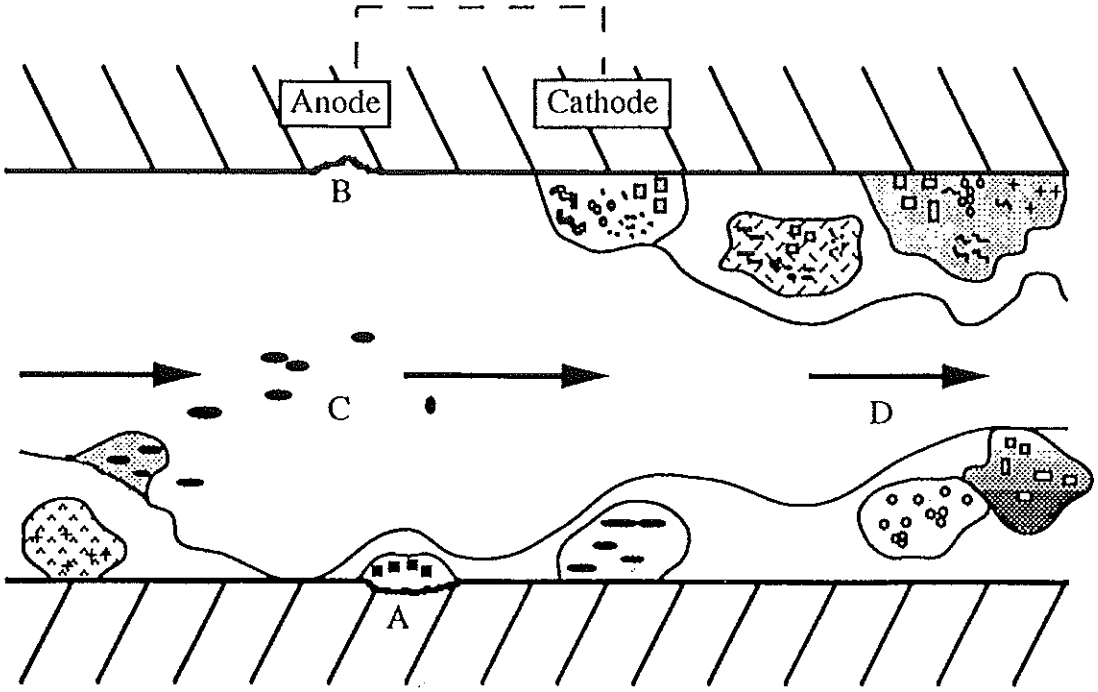


**FIGURE 1** A schematic diagram of biofilms in an industrial water system. Biofilms can cause surface deterioration due to bacterial processes (A) and the formation of a corrosion cell caused by the production of metabolites and biopolymers with different charges produced by adjacent microcolonies (B). Biofilms may be reservoirs for potential pathogenic bacteria that could detach from the microcolonies to contaminate the water system (C). Reduced efficiency of the pipes may be caused by physical blockage by thick biofilm formations (D).

attach to a wide range of man-made surfaces and grow as biofilms (Chapter 1.6). Indeed, any surface that is wetted or submerged in water, such as heat exchangers, cooling towers, water tanks, and pipelines within industrial processes, offers the possibility of microbial attachment and may result in surface deterioration, contamination of the industrial processes by potentially pathogenic/spoiling microorganisms, physical blockage of the conduit, and/or impairment of the function of the surface (Figure 1; Lappin-Scott and Costerton, 1989). It is important to eliminate or to control this microbial growth, as the resulting damage to the industrial process is often very costly. Several factors need to be considered before devising the relevant testing procedures.

## SCREENING OF ANTIMICROBIALS FOR INDUSTRY

Contamination of industrial waters often introduces microorganisms into environments that can provide growth nutrients to sustain their development. In considering methods to screen for novel antimicrobial activity and for the ability of developed strategies to control such growth, three specific factors are noteworthy:

1. Industrial waters may support the growth and/or survival of mixed communities of bacteria, and such growth may differ from that of similar organisms in monoculture.
2. If growth nutrients are not available then bacteria can exist in dormant forms, as spores or starved bacteria (Somnicells, Chapter 1.5).
3. Bacteria will generally attach to any available surface and grow as the sessile phenotype.

Each of these factors, relating to the survival and growth of bacteria within industrial waters, may affect their response to antimicrobial agents and treatment regimens. Each of these considerations will therefore be considered separately.

## Mixed Cultures

The efficacy of biocides is usually tested using pure cultures. This emphasis in microbiology originates in the work of Koch (1881). It is, however, generally considered that such techniques overlook the importance of communities of microorganisms which are comprised of mixed species (Bull and Slater, 1982). In natural environments bacteria are rarely found in monocultures. Even within extreme environments (in terms of pH, temperature, pressure, and salinity), mixed microbial communities are typical. Within mixed communities, the individual species interact and frequently derive benefit from their association with one another. Indeed such associations may extend their overall metabolic capability (Slater, 1981). For example, mecoprop, a phenoxyalkanoic herbicide, has a complex chemical structure and contains chlorinated substituents. To date there have been no reports of the ability of pure cultures to degrade this herbicide. Interacting communities of bacteria, however, are able fully to degrade mecoprop (Figure 2).

In industrial waters, microorganisms exist within mixed, interacting communities. Many of the problems associated with growth of microorganisms on pipelines and other metal surfaces are caused by interacting communities of bacteria. In order, therefore, to prepare relevant inocula for the testing of novel biocides for industry, it is necessary to use mixed cultures of bacteria rather than pure cultures, and to allow these to establish growth niches that replicate those *in situ*. The best method is to collect the water itself, together with scrapings from the contiguous surfaces, and use them as an inoculum into an appropriate *in vitro* growth model and testing system.

## Starved Bacteria

Most industrial processes use water either as a raw material or as a coolant. Generally a higher quality water is used as an ingredient than is required for cooling and other purposes (Geesey, 1987). Whatever the water quality, none of the processes involved is capable of maintaining a sterile environment. While most workers realize that even the highest quality waters may become

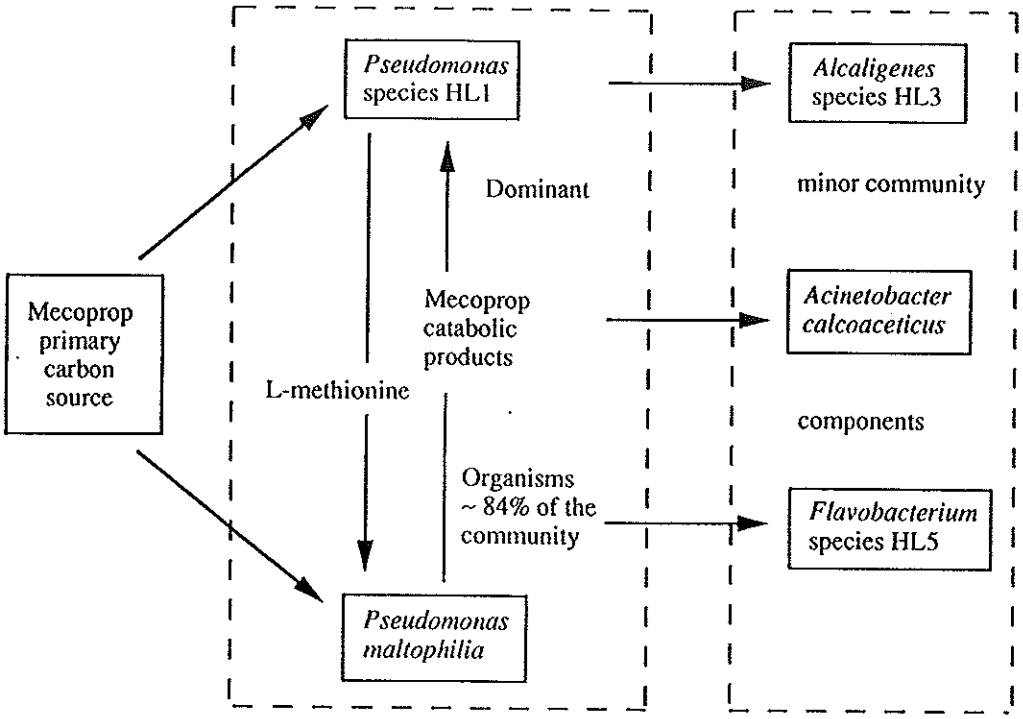


FIGURE 2 An example of a community of microoganisms interacting to degrade the herbicide, Mecoprop, in a natural soil environment. (Data from H. M. Lappin, Ph.D. Thesis, University of Warwick, U.K. 1984. With permission.)

contaminated, they often wrongly assume that such water is so pure that it will not support microbial growth or surface attachment involving active metabolism. In the absence of sufficient nutrients bacteria do not necessarily die. There is now a wealth of research describing survival of bacteria in low-nutrient environments. The reader is directed to a recent review on the subject of starvation survival (Kaprelyants, Gottschal and Kell, 1993) and also to Chapter 1.5 of the present volume. In essence, many bacteria do not die when all of the available growth nutrients have been utilized, rather they adopt a series of responses that assist their general survival (Novitsky and Morita, 1976; Chapter 1.3). These responses include the utilization of storage compounds, the formation of periplasmic spaces, breakdown of nonessential cellular components, reductions in endogenous metabolism, the production of specific proteins, reductions in cell size, and strategic reductions in population viability (Lappin-Scott and Costerton, 1990). In total these responses produce bacterial cells that are able to withstand starvation for indefinite periods. In many respects they are analogous to spores in that they are hardy structures which are able to resuscitate and grow when favorable growth conditions are reestablished.

An important consequence of the starvation-survival response in bacteria that contaminate industrial processes is that such organisms respond to antimicrobial agents in a manner completely different from that of their vegetative counterparts. Most of the chemical antimicrobial agents currently used to kill industrial contaminants have been developed against vegetative bacteria. If

starved bacteria have a different response from that of the vegetative bacteria then they may survive such treatments. Studies of the efficacy of antibiotics and biocides have observed a reduced rate and extent of killing of slow-growing bacteria (Brown, Allison and Gilbert, 1988). To date, however, the ability of industrial biocides to eradicate starved bacteria has been little studied.

One recent investigation, recently undertaken in our laboratories, has involved the sulfate-reducing bacteria (SRB). Within the oil industry, SRB are nuisance organisms in that they grow within subterranean rock formations and the pipelines of the topside facilities to produce hydrogen sulfide, a toxic, corrosive gas (Hamilton, 1985). Thermophilic SRB, isolated from North Sea oil production waters, are able to exist as starved, dormant cells within seawater, but become fully resuscitated when given growth nutrients (Lappin-Scott, Bass and Sanders, 1992; Bass, Lappin-Scott and Sanders, 1993). Lappin-Scott and Sanders (1993) exposed vegetative and starved forms of SRB to different concentrations of glutaraldehyde. The effectiveness of the treatment was measured by monitoring metabolic activity over a 24 h post-treatment period. The metabolic activity of the vegetative cells decreased rapidly following exposure to glutaraldehyde, whereas that of their starved counterparts remained at over 50% of the basal level. This study demonstrated that even a chemically highly reactive biocide such as glutaraldehyde is not effective at eliminating starved bacteria and emphasised the need to consider the nutritional status of bacterial cells prior to preparing challenge inocula for biocide testing. If biocides are developed only against vegetative rather than starved bacteria, then their effectiveness in the field will be unpredictable.

## Sessile versus Planktonic

The attachment of bacteria to surfaces has been dealt with in detail in Chapter 1.7 of this volume. This topic, therefore, will be only briefly dealt with in the context of industrial processes.

Bacteria grow within industrial tanks, pipes, and machinery, and often contaminate the industrial processes themselves. Growth of bacteria on heat exchangers reduces the efficiency of the cooling surfaces, with associated financial losses to industry, by acting as an insulating layer (McCoy, 1987). An additional problem, also responsible for huge financial losses, is that of microbially induced corrosion within industrial pipelines and tanks, and is responsible for huge financial losses. The bacteria growing on these surfaces, and in their physical proximity, promulgate chemical reactions between the bacteria and the substratum. Thus, during biocorrosion, SRB reside within the anaerobic regions of the biofilm and establish electrochemical corrosion cells between the biofilm and the surface metal. Again, suitable test methods must be deployed which duplicate such growth and provide inocula which are appropriate for effective biocide development against both the sessile and the planktonic phenotype.

## TEST METHODS

Historically, the testing and screening of antimicrobial agents has been undertaken using pure cultures of vegetative, planktonic bacteria which have been grown in nutritionally rich media. Since this is atypical of most natural environments, then these testing methods screen antimicrobial agents only for their relative activity. The data generated cannot be extraploated to give direct information on *in situ* activity. Thus, compounds with activity against only sessile populations will be overlooked and compounds with especially good activity against fast-growing planktonic cells might be wrongly selected for further development.

In response to such problems, different methods have been developed to screen and test agents against microbial physiologies and communities which more closely resemble those found *in situ*. Each of the various testing methods uses different procedures to establish and maintain the test biofilms. Accordingly, each approach produces biofilms that are representative of different ecological niches and differ from one another in terms of physiology and metabolism. The established procedures include the use of reactors, such as the RotoTorque and the Constant Depth Film Fermenter, and biofilm sampling systems, such as the Robbins device, and the insertion of coupons within batch and continuous cultures. The application of methods, such as the perfused biofilm fermenter (Gilbert et al., 1989), which offer the possibility of greater control over the expressed phenotype, have been described in earlier sections (Chapters 1.7 and 3.7). All of these experimental approaches exercise different levels of control over biofilm formation and growth under conditions which model a number of environmental niches. These factors probably have a significant impact on the efficacy of the biocides tested.

In general, biocides to control contamination in industry should be tested in the context of their intended use; that is, if they are to be used against surface-attached bacteria, they should be tested against cells grown in this manner. The testing procedure should model the real situation as closely as possible. These considerations should influence the choice of test procedure, and we now briefly review the main approaches. We have described three factors that we consider important in developing suitable inocula for test methods, namely the use of mixed cultures representing *in situ* and growing both as sessile and planktonic populations under low nutrient or starvation conditions. Such organisms must therefore be used as the initial inocula for establishing the following *in vitro* test systems.

### Biofilm Reactors

#### RotoTorque® Biofilm Reactor

The RotoTorque, a rotating annular reactor, produces biofilms on surfaces which are subject to different frictional resistance to attachment and maintains

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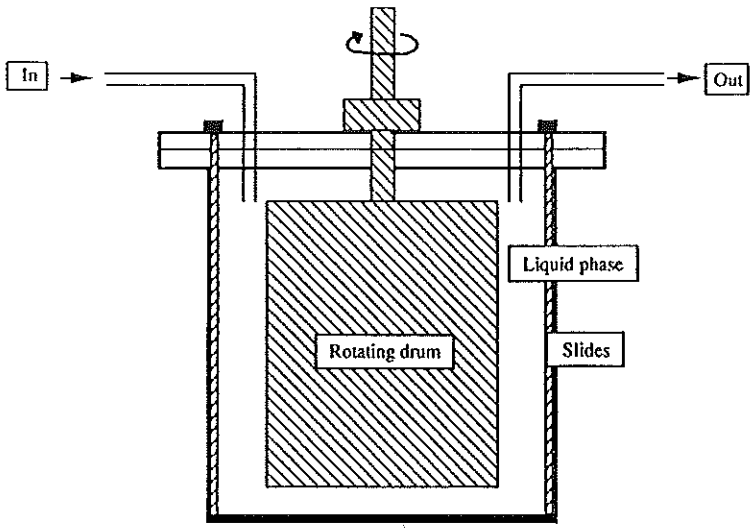


FIGURE 3 A diagram of the RotoTorque, a continuous flow reactor for forming biofilms under different shear stress. It is composed of an inner rotating drum and an outer glass cylinder into which glass slides are inserted, separated by a liquid phase containing microbial communities.

conditions which approximate to an open, continuous culture (Characklis et al., 1982). The RotoTorque consists of a rotating inner drum and a stationary outer drum operating as a continuous flow chemostat reactor (Figure 3). The outer drum contains 4 to 12 removable slides as an integral part of the inside wall. The slides permit sampling of the biofilm within the reactor. Fluid is recycled through the reactor to maintain a constant fluid shear stress which is independent of the nutrient flow into the system. These reactors maintain biofilm thickness by the controlled application of frictional force. The bulk fluid is kept to a minimal level such that planktonic growth is negligible and the bulk of the metabolic activity within the reactor can be attributed to the sessile population (Characklis, 1990).

Biofilm accumulation within the reactor can be monitored indirectly by measuring the drag force on the inner rotating cylinder. This is monitored by a torque transducer mounted onto the shaft between the motor and the cylinder. When the fluid shear stress in a clean reactor is calculated, the change in the fluid shear stress when a biofilm is present provides information about biofilm accumulation. Conversely, the effect of biocide treatment upon the extent of biofilm development can be easily monitored by the reduction in force required to turn the inner cylinder (Characklis, 1990). In a study by Characklis et al. (1980), periodic chlorination treatment of a biofilm showed that a temporary drop in frictional resistance occurred after treatment. This indicated that, during chlorination, there was a partial detachment of biofilm but that the biofilm recovered and built up before the next chlorination treatment.

This biofilm generator is well suited for systems where variable and high fluid shear stress is a major factor during biofilm formation. Industrial situations where this would be considered important are recirculating water systems such as pumps, cooling plants, and swimming pools.

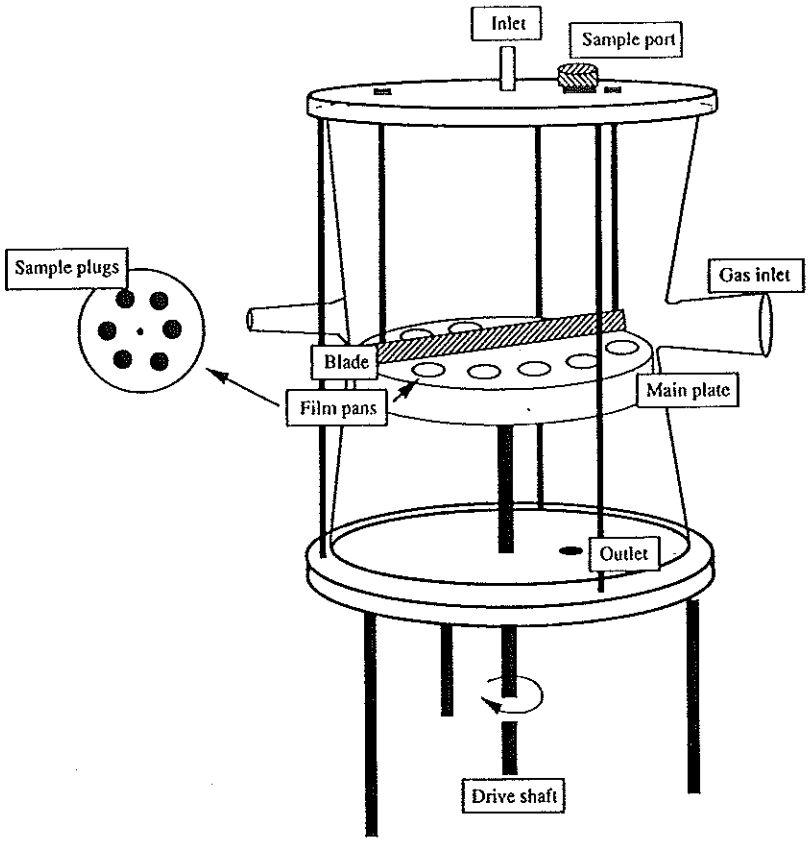


**FIGURE 4** The constant-depth biofilm fermenter forms biofilms of a predetermined thickness by scraping excess biofilm with a blade. The fermenter is constructed with a main plate containing a number of film pans into which six surfaces have been recessed. A blade is held stationary over the surface while the main plate rotates beneath allowing the biofilms to be scraped off. Medium is fed to the biofilms by a port in the top of the fermentor and an outlet is at the bottom.

## Constant Depth Film Fermenter

The Constant Depth Film Fermenter has been used by Coombe, Tatevossian, and Wimpenny (1982) and was further developed by Peters and Wimpenny (1988) as a method for studying the formation and treatment of dental plaque. The fermenter has also been applied to the study of biofilm formation by freshwater communities. The main features of the Constant Depth Film Fermenter are that it maintains a biofilm, contained within a recessed chamber, at a predetermined and constant depth by mechanically scraping the cells from the surface. The biofilm is developed onto one of six surfaces recessed into a film pan (Figure 4). There are 15 film pans on a main disk within the fermenter. The main disk, driven by a motor, rotates beneath a stationary, angled Teflon® blade which scrapes excess biofilm from the top of the film pans (Wimpenny, Peters and Scourfield, 1989). Nutrient is fed through an inlet at the top of the fermenter leading down to the surface of the rotating main disk where it is dispersed uniformly by the blade to the biofilms. Excess and spent medium and

biomass is voided through the base of the fermenter. The top of the fermenter has a sampling port through which film-pans can be removed aseptically for sampling. Mixed community dental biofilms have been developed, using this system, to a thickness of 20 mm using mercury amalgum hydroxyapatite and polymethylmethacrylate resin as colonizing surfaces (Wimpenny, Peters and Scourfield, 1989).

Kinniment and Wimpenny (1990) used the Constant Depth Film Fermenter to determine the response to various biocides of model biofilms formed from microbial communities isolated from contaminated cutting fluids. They used viable counts and protein content to monitor the development of the biofilms. A concentration of 200 ppm of formaldehyde added to a biofilm formed over 50 h caused a decrease in both the protein content and viable cell density, within the biofilm, relative to untreated biofilm controls. The Constant Depth Film Fermenter provides a reproducible system for biofilm formation of a predetermined thickness, so that biocide efficacy can be tested. This system would be used for studying very thick biofilms such as dental plaque, sludge, and some industrial water systems.

## Biofilm Sampling Devices

### The Robbins Device

The Robbins device was first developed to provide multisampling facilities for the study of biofilm formation in tubular systems (McCoy et al., 1981; Ruseska et al., 1982). The tubular structure, made of admirality brass, contains stainless steel studs which screw into the device (Figure 5). Each stud contains a removable test surface of 0.5 $cm^2$ from which the developed biofilm may be sampled for viability determinations and electron microscopy. The Robbins device is well adapted for use as a self-contained laboratory experiment but may also be fitted into the plumbing of industrial water systems (Figure 5) to monitor the performances of biocides *in situ*. By forming a biofilm within the Robbins device and then treating with different regimens of biocide, the survival of sessile bacteria and the efficacy of the treatments could be determined. These investigative procedures involve both laboratory-based studies of single or groups of biocides against sessile cells and *in situ* observations of biocide efficacy. Continued on-line monitoring of the biofilm show the effectiveness of the biocide, even after treatment is discontinued. With a large number of sample ports, time studies of the effects of biocide treatment can be undertaken (Costerton and Lashen, 1984).

The Robbins device was modified in order to facilitate its routine use for the laboratory testing of biocides and antibiotics (Nickel et al., 1985). Constructed from a perspex (Plexiglas) block, the modified Robbins device has a lumen through which a culture can be passed and contains 25 equally spaced sample ports into the lumen. The ports can receive studs, the faces of which form a

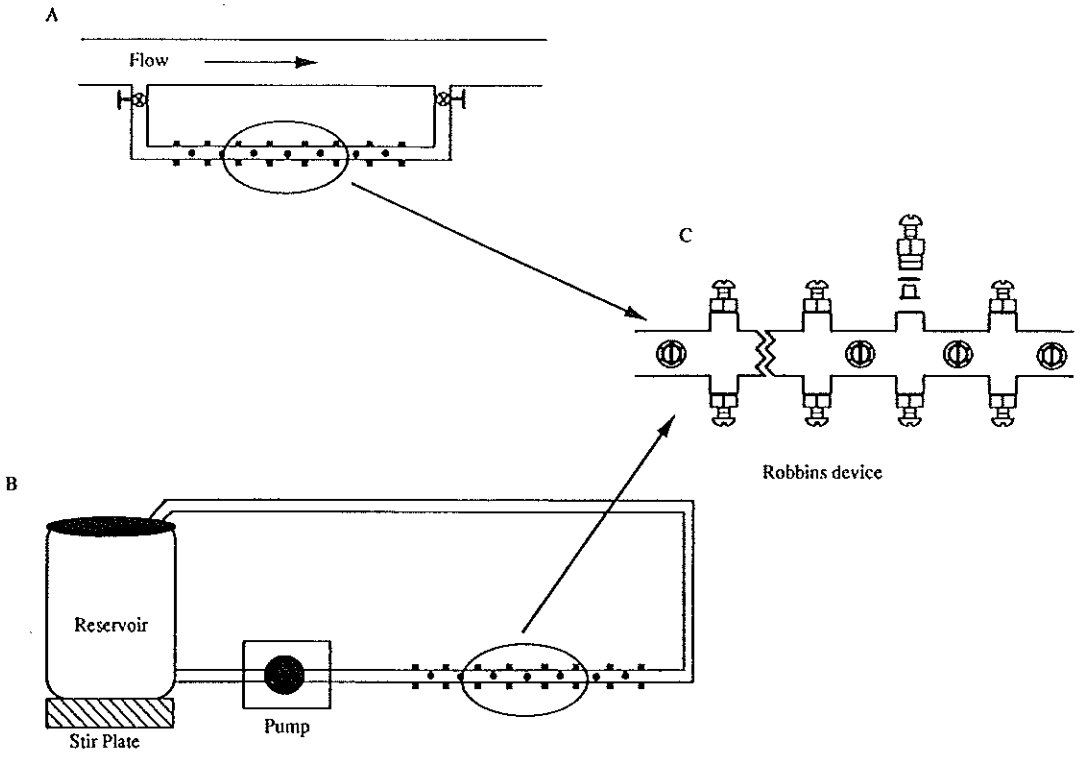


**FIGURE 5** A schematic diagram of a Robbins device (C) showing a pipe with screws holding surfaces within the pipe. The system is strong enough to be used both within industrial plumbing systems where biofilm samples can be observed by redirecting the flow through the Robbins device rather than the main pipes (A). The Robbins device can also be used within the laboratory attached to a reservoir the culture pumped through via a peristaltic pump (B).

surface continuous with that of the lumen. Without removing the surfaces and potentially disrupting the biofilms, the sessile cells on the stud surfaces can be treated with antibiotics or biocides by pumping the solution through the lumen together with nutrient medium. Samples of the biofilm can be removed aseptically and, again without disrupting adjacent biofilms, assessed for bacterial viability. By substituting different materials onto the tips of the studs the effects of biocides against biofilms formed onto different surfaces can be assessed. Modified Robbins devices and other forms of tubular sampling devices have been attached to either batch cultures (Nickel et al., 1985) or chemostats (Camper, 1993; Green, 1993; Hoyle, Williams and Costerton, 1993; Jass, Sharp and Lappin-Scott, 1992) as a source of their inocula.

Tubular biofilm sampling devices of a similar design have been used by other researchers to monitor biofilm formation and biocide activity. For example, Green and Pirrie (1993) used different tubes as surfaces, connected to either a batch or a chemostat culture containing *Legionella* spp. By removing sections of the tubing and placing them into different biocide solutions, the efficacy of the biocides against the *Legionella* could be analyzed at different concentrations. This system also proved effective at comparing different biocides against sessile cells.

### Submerged Test Pieces

Many researchers have used chemostats to control the growth of planktonic bacteria (Anwar et al., 1989; Keevil et al., 1987; Walker et al., 1991). Computer monitored chemostats provide full, automated control over the planktonic culture and thus provide reproducible planktonic inocula for biocide testing. By inserting a selected surface into a chemostat, biofilms will form on the test piece in a controlled and reproducible manner. Although the biofilm still maintains an heterogeneous environment, the growth rate of the planktonic cells can be maintained. Furthermore, for mixed cultures, species composition may be kept constant for the biofilm formation. There have been different chemostat set-ups to accomplish this. For example, Keevil et al. (1987) and Keevil, Mackerness, and Colbourne (1990), formed mixed consortia dental plaque biofilms on glass tiles by using a two-stage chemostat. The first stage provided the seed culture for the second stage. The test surfaces were inserted into the second stage for up to 28 days before sampling. Use of a two-stage fermenter ensures that the primary fermenter, which generates the inoculum for biofilm formation, is never exposed to the test conditions/substances. Using this approach they tested the biocide monochloramine, against both biofilm and planktonic cells by adding 0.2 mg/ml monochloramine to the fresh medium fed into the second stage of the chemostat. They reported that the biocide reduced both the biofilm and planktonic consortia by one order of magnitude (Keevil, Mackerness and Colbourne, 1990). Furthermore, not only was the biofilm community different after the treatment with the biocide, but the planktonic community was also different from the biofilm. Such systems allow simultaneous testing against both biofilm and planktonic cultures and facilitates comparative studies of the efficacy of biocides against each phenotype. Such studies reinforce the importance of testing biocides against mixed community biofilms rather than isolated monocultures.

## CONCLUDING REMARKS

It is important that biocides and antimicrobial agents be properly assessed for their effectiveness before application in industrial or medical situations. The use of procedures based on pure cultures of vegetative, planktonic bacteria are unreliable in that they are not typical of the environments in which the biocide will be applied. The appropriate procedures involve the use of random samples of inocula obtained from the environment in which the biocides are to be used. Such a testing procedure may be more expensive than those using pure cultures, as biocides may have to be tested against a variety of inocula reflecting the variety of situations in which the biocide is expected to be effective. This might be a reasonable price to pay for improved effectiveness in use.

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