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REVIEW

In vitro models for oral malodor

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A model is a representation of some real phenomena and contains aspects or elements of the real system to be modeled. The model reflects (or duplicates) the type of behavior (or mechanisms) seen in the real system. The main characteristic of any model is the mapping of elements or parameters found in the system being studied (e.g. tongue dorsum biofilm *in situ*) on to the model being devised (e.g. laboratory perfusion biofilm). Such parameters include correct physico-chemical (abiotic) conditions as well as biotic conditions that occur in both model and reality. The main purpose of a model is to provide information that better explains the processes observed or thought to occur in the real system. Such models can be abstract (mental, conceptual, theoretical, mathematical or computational) or 'physical', e.g. in the form of a real disaggregated *in vitro* system or laboratory model. A wide range of different model systems have been used in oral biofilm research. These will be briefly reviewed with special emphasis on those models that have contributed most to knowledge in breath odor research. The different model systems used in breath odor research are compared. Finally, the requirements for developing an overall 'bad breath model' from considering the processes as a whole (real oral cavity, substrates in saliva, biotransformation by tongue microflora, odor gases in the breath) and extending this to the detection of malodor by the human nose will be outlined and discussed.
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Introduction

What do we mean by the term 'model'?

A model is a representation of some real phenomena and contains aspects or elements of the real system to be modeled. The model reflects (or duplicates) the type of behavior (or mechanisms) seen in the real system. The main characteristic of any model is the mapping of the elements or parameters found in the system being studied (e.g. tongue dorsum biofilm *in situ*) on to the model being devised (e.g. laboratory perfusion biofilm). Such parameters include correct physico-chemical (abiotic) conditions as well as biotic conditions that occur in both model and reality.

Why do we need models?

The main purpose of a model is to provide information that better explains the processes observed or thought to occur in the real system. Their main value is therefore explanatory power or predictive capability. With regard to oral biofilms, their complexity is so large that it is difficult to study them without recourse to an experimental laboratory model. To study mechanisms or processes occurring in biofilms, it is necessary to perturb them. There is a limit to the amount of perturbation that can be done on a real tongue biofilm. Moreover, it is difficult to keep all other environmental factors controlled or constant. It is easier to carry out experiments in the confines of a well-equipped laboratory than to work in the human mouth. Moreover, in a laboratory system the environmental conditions can be controlled more precisely and the perturbation of the system can be controlled at will. This degree of control allows the operator to vary one particular parameter at a time and observe the resultant effects (called a 'unifactoral' approach). The response of the biofilm system can be determined across a wide range or set of environmental conditions and cause–effect relationships proposed, as clear answers to proposed questions or hypotheses. All the other components of the system are held constant or simply ignored. If the model turns out to be a good representation of, say, oral biofilm growth and metabolism it can then be used for other purposes such as screening compounds with interesting biological activity against biofilms (e.g. potential anti-plaque, anti-odor or anti-caries compounds) and predict their likely effects if given *in vivo*.

Types of model (physical and abstract)

Models can be abstract (mental, conceptual, theoretical, mathematical or computational) or 'physical', e.g. in the form of a real *in vitro* system or laboratory model. Even

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the oral cavity itself can be regarded as a model if perturbed and measured (or monitored) in a meaningful way.

Physical model
In terms of complexity, the physical (experimental) model may range from the most complex; *in situ*/*in vivo* (real oral cavity) to less complex (e.g. *in vitro* perfusion biofilm) to least complex (e.g. *in vitro* test tube reaction). Physical models may also be: static/dynamic, open or closed, steady-state or non-steady-state (see later examples).

Theoretical model (conceptual, mathematical and computer)
In practice, many practical or empirical models have theoretical counterparts that improve the explanation and predictive powers of the system. As an explanatory system a model is nearly always only partial (explaining just some of the processes or main steps) rather than encompassing the process *in toto*. All the conceptual ideas we have on biofilms are bound to be less than reality, so our conceptualizations can themselves be considered to be models in the abstract sense of the word (a so-called mental model). Even the theoretical models can be of different types. For example, a model may be deterministic (average behavior of the system) or stochastic (the collective and singular behaviors of the small elements or units – cells or molecules – that help make up the system using probability theory).

Black box vs white box
Most systems contain collections of interacting subsystems. With respect to the whole the parts are seen as subsystems. With respect to the parts, the whole is seen as a supersystem. It is possible to measure the overall behavior of the supersystem as a whole (by just considering its total input and total output), without being aware of all its parts or which part of the input goes to which subsystem. This approach considers the system as a 'black box', something that takes an input, and produces an output, without considering what happens in between. If in contrast all the system's internal processes are revealed, it can be called a 'white box'. Although the black box view may not be completely satisfying, in many cases this is the best we can get as many processes are still unknown and the only thing that can be clearly established is the final result. The 'black box' view is sometimes good enough but this may depend on the hypothesis set and the nature of the known/unknown processes. Most systems are gray boxes (elements of black and white). The black box view is not restricted to situations where the internal systems are unknown as even when known (or knowable) we may prefer to ignore the internal details. For example, when modeling volatile sulfur compound (VSC) production in biofilms, it may not matter which particular microbe produces VSC. It may be sufficient simply to know the total amount of substrate (cysteine) that enters the biofilm to estimate the total amount of VSC produced. The 'black box' view of the biofilm will be much simpler and easier to use for predictive calculation of overall VSC levels than the more detailed 'white box' view, which might trace the movement of every portion of substrate to every particular microbial cell in the system.

These two complementary views, 'black' and 'white', of the same system illustrate a general principle: systems are structured hierarchically. They consist of different levels. At the higher level, you get a more abstract, encompassing view of the whole, without attention to the details of the components or parts. At the lower level, you see a multitude of interacting parts but without understanding how they are organized to form a whole.

Computer-based or mathematical modeling
Using computers it is now relatively easy to summarize quantitatively certain outputs for a range of given inputs based around some mathematical equation. An example is the logistic equation for predicting biofilm growth:

$$\ln\left(\frac{k-x}{x}\right) = a - rt,$$

where x = biomass, a = constant, k = upper limit constant, r = growth rate constant and t = time.

However, this is a black box empirical approach with two or more unknown constants without any real world meaning. They do not relate to simple measures with real units of measurement. They are simply a series of mathematical descriptions of curves rather than a description of the real mechanisms. Although empirical systems can be made more complicated using more complex algorithms they bring little in the way of understanding and have limited use.

A true alternative is to build the model around more basic theoretical-mechanistic equations: e.g. the equations relating the relationships between reaction rate (or growth rate), substrate (or limiting nutrient) concentration and enzyme (or cell) affinity for substrate (Figure 2). This includes the Michaelis–Menton equation for enzyme reactions and Monod equation for whole cell transformations and growth. The clear advantage of the Michaelis–Menton or Monod model is that the parameters (V_{max}, V, s, K_m or μ_{max}, μ, s, K_s respectively) have real biological meaning and are experimentally accessible.

Any set of mathematical equations may constitute a mathematical model provided they aim at representing a real system and are based on some theory regarding the systems operation and are not just made to fit it in a purely empirical way.

Mathematical models that regard biofilms as homogeneous steady-state films containing a single species have been proposed (Rittmann and McCarty, 1980). This model has been evolved to cover dynamic multisubstrate-multispecies biofilm computer models (Wanner and Gujer, 1986; Rittmann and Manem, 1992; Wanner and Reichert, 1996). An approach using discrete cellular automata (to simulate the rules that govern the lives of microbial cells) has also been

employed to model biofilms (Wimpenny and Colasanti, 1997; Picioreanu *et al*, 1998). These allow the simulated biofilm structure to evolve as a self-organization process, emulating how real bacterial cells organize themselves into biofilms. These models produce realistic, structurally heterogeneous biofilms. Mathematical models of diffusion/reaction in dental plaque (incorporating Fick's law of diffusion) have also been described (Dibdin, 1981, 1997) as have models to predict the effects of antimicrobial activity within biofilms (Dibdin *et al*, 1996; Stewart, 1996).

Modeling terminology

In developing, constructing and using models to explain, predict and ask questions of real life processes, it is of fundamental importance that the purpose of, and assumptions underpinning, the model are clearly stated and understood. In addition, the assumptions made should be considered and their potential impact questioned whenever the model is used.

However, it is important that modeling strategies and associated procedures are clearly defined and adopted so that the quality and robustness of model output can be quantified and limits of applicability set. A vast body of literature, particularly for conceptual and computer-based models, has been written concerning the criteria that should be adopted in order to signify the usefulness of a given model. The concepts promulgated in such studies are transferable to use with 'physical models' and, as such, some especially important concepts are summarized in Table 1.

Table 1 Modeling terminology and procedures

Concept/term	*Description/process*
Reality	An entity, situation or system that has been selected for analysis (Schlesinger, 1979)
Conceptual model	Verbal description, equations, governing relationships or 'natural laws' that purport to describe reality (Schlesinger, 1979)
Verification	The process of ensuring that the implementation of the conceptual model is correct (Schlesinger, 1979) Substantiation that a model is in some sense a true representation of a conceptual model within certain specified limits or ranges of application and corresponding ranges of accuracy (Refsgaard and Henriksen, 2003)
Validation	The determination that the theories and assumptions underlying the conceptual model are correct and that the model representation of reality is reasonable for the intended use of the model (Sargent, 1988) Substantiation that a model, within its domain of applicability, possesses a satisfactory range of accuracy consistent with the intended application of the model (Refsgaard and Henriksen, 2003)
Turing test	The process whereby experts who are knowledgeable about the system in question (reality) are asked if they can discriminate between system and model outputs (Sargent, 1988)
Credibility	That there is a sufficient degree of belief in the validity of the model to justify its use for research and decision-making (Rykiel, 1996)

Considerations in modeling biofilms

1. Existing knowledge of the natural system being modeled: in our case we are interested in modeling tongue biofilm in terms of its growth and production of volatile compounds, particularly VSCs. The biofilm can be defined as a matrix-embedded microbial population where cells are adherent to each other and/or to other surfaces and interfaces. There are different forms of biofilm in respect of microbial composition and cell density distribution. For example, biofilms may range from patchy microcolonies of one or two species toward open spongy structures of microcolonies and communities with pores and channels, toward dense biofilms with highly packed bacteria. Unlike biofilms that form on hard surfaces, the tongue biofilm is thought to be relatively porous with respect of ingress of substrates and egress of products. Although there is a large amount of work published about VC/VSC production from various microbial species when fed different substrates there has been very little published about tongue biofilm structure and its properties (growth rate, biotransforming capacity) *in situ*. It is likely that in common with all biofilms, the tongue biofilm mode of existence will protect microbial cells against biocides and antibiotics and may be over 100 times more resistant than their planktonic counterparts.

2. Objectives: what it is that you want to find out about. Laboratory models enable the researcher to have control over: types of microbes, environment, nutrient supply and substrata. There are also increased options for experimental protocols, sampling and analysis. Different models are suitable for different purposes.

3. Biodiversity classes (Sissons *et al*, 1999) and source of microbes: class I: single species (monospecies) (e.g. *Staphylococcus epidermidis* on catheter); class II: 2–5, 6 or 7 (e.g. gram-negative species on urinary tract catheter); class III: 7–30 or so (eg. *Bioremediation consortia*); class IV: 30–500 or so (e.g. dental plaque); class V: 500–1000 or so (e.g. soil communities on clay particles). A microcosm is a laboratory subset of the natural population from which it originates. It retains the genetic, temporal and structural heterogeneity of the natural system and can evolve further in response to environmental changes.

4. Matching conditions in model and reality (mapping of biotic and abiotic conditions). In order for an experimental model to closely resemble and behave like the real biofilm *in situ* there is an obvious need to map the chemico-physical environment (temperature, pH, nutrients and inhibitors). The abiotic (chemico-physical) parameters include: temperature (35–37°C), pH (6.5–7.5), osmolarity (equal to that of saliva), type and concentrations of nutrients or substrates and type and concentrations of inhibitory compounds. For experimental purposes we can use a spectrum of nutrient molecules from one main carbon/energy (C/E) substrate (e.g. glucose) to a complex mix containing sugars, polysaccharides, amino acids, peptides, and proteins. Moreover, the medium could be synthetic-defined or complex (e.g. artificial saliva *vs* real saliva filtrate).

The biotic parameters include the diversity of living entities and again this can be a spectrum from monoculture to real inoculum (complex microcosm).

5. Growth rates and cell densities similar to those *in situ* (oral cavity).

6. Fluid regime (pulse or dose, defined or undefined media) and hydrodynamics (flow and shear: turbulent, plug and laminar). The fluid regime will also include treatment protocols (antimicrobials, inhibitors of VSC production).

7. Substrata and conditioning film: important if studying initial formation of biofilms. Hydroxyapatite and salivary pellicle might be considered for plaque biofilm whilst salivary-coated polymeric material could be used to simulate the tongue surface.

8. Sampling protocol: e.g. number of replicate samples; pre-, postperturbation or continuous monitoring?

9. Analytical techniques (measurements of pH, reducing power, metabolic end-products, substrate utilization and enzyme assays). Discontinuous samples or continuous (real time) monitoring.

Models currently used in oral biofilm research

There are a range of laboratory experimental physical models used to study oral microbes and biofilms, from simple batch culture closed systems (using test tubes, closed flasks or wells in microtiter plates) to open, continuous flow arrangements. *In vivo* or *in situ* experiments (e.g. studying VSC production following cysteine rinses) may relate well to the natural situation and reveal important information. However, only limited measurements and perturbations are possible in a real human mouth. Computer-based modeling using inputs based on real empirical data can summarize quantitatively what is known about biofilm properties and may give useful insights to help generate and test hypotheses. For studying biofilms in general and oral malodor in particular, a number of different models or systems have been used *in vitro*; some of these will be briefly described.

Closed systems

1. Incubated saliva or salivary sediment or incubated species of selected microbes as cultures. These experiments tell us something about the potential of species and/or microcosms to do particular types of transformation. It is clear from previous work using closed systems (Tonzetich, 1971; McNamara *et al*, 1972; Tonzetich and McBride, 1981; Kleinberg and Codipilly, 1995) that anaerobes, and particularly the gram-negative anaerobes (GNA), tend to produce a higher degree of malodorous VC/VSC in culture than other groups. By incubating species of oral microbes in liquid culture containing elevated levels of amino acid substrates, Kleinberg and Codipilly (1995) were able to demonstrate odor production by a range of species against a range of substrates. In general, gram-positive species were not particularly effective at producing odor whilst gram-negative species, particularly the anaerobic species *Porphyromonas gingivalis* and *P. intermedia*, were effective odor producers. The most effective substrates inducing odor were cystine/cysteine, methionine, ornithine, lysine and tryptophan. *Porphyromonas gingivalis* produced increased odor with all these substrates which implies that it may have the potential to produce hydrogen sulfide, methyl mercaptan, putrescine, cadaverine and indole from respective substrates. A more objective analysis of VC and VSC production by oral species has been made using gas chromatography mass spectrometry measurements of culture headspace (Kostelc *et al*, 1980; Greenman, 1999) and data show that a wide variety of volatile chemical species are produced from a wide range of species/samples.

Batch cultures are simple and may be adequate for gaining certain types or levels of knowledge but they have major limitations. In batch culture, the whole physico-chemical environment (number of cells, concentrations of substrates, nutrients, products, pH and oxygen tension) is constantly shifting as an inevitable consequence of growth in a closed system. The conflated variables disallow strict cause–effect relationships to be discerned between set environmental conditions and measured physiological responses.

2. Cell suspension assay (non-growth system; initial rates measured using defined assay). Provided these assays are of short duration they can give quantitative rate data (K_m, V_{max}) using fresh samples (e.g. tongue scrape material). In contrast to the previous system they are suspension assays not involving growth/culture and are generally more useful than the previous model.

Open systems (continuous flow devices)

Table 2 describes a number of different systems that have been used in the past and the reader is referred to original papers (Table 2) to find out more details.

Chemostat

The chemostat is an ideal device for generating steady-state homogenous cultures and has been used widely in oral microbiology research (Marsh *et al*, 1983; McKee *et al*, 1985; McDermid *et al*, 1986), including research on oral malodor (Greenman, 1999). However, its use for studying biofilms is very limited as it operates on the principle that all cells remain in suspension (planktonic growth) and if biofilms do form within the system, they disturb the steady-state. Nevertheless, chemostats are often used to provide steady-state cell suspensions to subsequently feed into a flow device (Herles *et al*, 1994; Li and Bowden, 1994). A two-stage chemostat system with test surfaces immersed into the second-stage vessel has been applied to the study of biofilm formation (Keevil *et al*, 1987; Keevil, 1989; Marsh, 1995).

Flow cell systems

Continuous flow cell (slide) models have been used widely (Table 2) to study biofilm structure, especially initial events of formation. They often employ microscopy such as confocal laser scanning microscopy to capture and analyze images over time. Although useful for studying attachment and early stages of growth and ecology they are not particularly suitable for studying

Table 2 A selection of *in vitro* biofilm systems (some applied to oral biofilms; a few applied to malodor research)

Biofilm system	*Applications*	*Authors*
Artificial mouth models using enamel or hydroxyapatite surfaces	Colonization, biofilm metabolism, effects of antimicrobial compounds	Russell and Coulter, 1975; Coulter and Russell, 1976; Dibdin *et al*, 1976; Sissons and Cutress, 1987; Zampatti *et al*, 1994; Sissons, 1997; Soukos *et al*, 2000
Calgary biofilm device	Antimicrobial susceptibility	Ceri *et al*, 1999; Spoering and Lewis, 2001
Robbins device	Antimicrobial and biocide efficacy	McCoy *et al*, 1981; Gristina *et al*, 1987; Hoyle and Costerton, 1991
Modified Robbins device	Antimicrobial susceptibility	Khardori *et al*, 1991; Larsen and Fiehn, 1995; Yassien *et al*, 1995
Continuous culture flow cell chambers	Mixed biofilm cultures and metabolic interactions; formation, ecology, cell death; efflux pumps; antimicrobial susceptibility;	Keevil *et al*, 1987; Herles *et al*, 1994; Li and Bowden, 1994; Wolfaardt *et al*, 1994; Marsh, 1995; Møller *et al*, 1998; Heydorn *et al*, 2000; De Kievit *et al*, 2001; Neu *et al*, 2002
Fluidized bed environments and airlift reactors	Biofilm structure; biotransformations	Gjaltema *et al*, 1995; Tijhuis *et al*, 1996
Perfused biofilm fermenter	Antimicrobial susceptibility; cell surface hydrophobicity; extracellular polymeric substances (EPS) production	Gilbert *et al*, 1989; Allison *et al*, 1990a,b, 1999; Duguid *et al*, 1992a,b; Evans *et al*, 1994
Constant depth film fermenter	Biofilm formation, ecology; effects of antimicrobials (including effects on oral malodor)	Kinniment *et al*, 1996; Wilson, 1996; McBain *et al*, 2003a,b,c; Pratten *et al*, 2003
Sorbarod system	Characterization of pure and mixed species biofilms; antimicrobial susceptibility; effects of cysteine on VSC (oral malodor)	Hodgson *et al*, 1995; Budhani and Struthers, 1997; Foley and Gilbert, 1997; Gander and Gilbert, 1997; Gander and Finch, 2000; Maira-Litrán *et al*, 2000; Greenman *et al*, 2002; Nelson *et al*, 2003; Spencer *et al*, 2003
Immersion of surfaces in chemostat cultures	Antimicrobial susceptibility	Anwar *et al*, 1989; Keevil, 1989
Rotating annular reactors, rototorque and biodrum	Colonization, ecology (shear stress)	Gjaltema *et al*, 1994; Arcangeli and Arvin, 1995; Allison *et al*, 1999; Lawrence *et al*, 2000

VC/VSC biotransformations so are generally not used in malodor research.

Larger-scale models

The constant-depth film fermenter (CDFF) has been widely used in studying biofilms (Table 2). In essence, this system allows biofilms to form in small recesses which are periodically swept with a blade to remove any material above the level of the recess. In this way it keeps the biofilm at a constant depth. All other physico-chemical parameters (medium, flow rate, atmosphere, temperature and pH) are carefully controlled. Although this system has many advantages over flow cells (especially for studying the properties of mature biofilms and the effects of substrates and antimicrobials) it has one major disadvantage; the growth rate of cells is ill-defined. Two parameters contribute to the growth of the biofilms; growth from within and further attachment of cells from without. This model is not able to separate the contributions made from these two parameters. Nevertheless, CDFF have been used to study the effects of inhibitors on VSC production by mixed culture microcosms of oral origin (Pratten *et al*, 2003).

Perfusion systems

Membrane bioreactors have been described elsewhere (Helmstetter and Cummings, 1963). The perfused biofilm fermenter (Gilbert *et al*, 1989) incorporates a bacterial cell impermeable membrane (e.g. Swinnex filters produced by Millipore Corporation) which is fed from one side with sterile growth medium. The microbial cells are attached to the other side of the filter (the underside). The attached microbes grow at a rate depending on the composition and flow rate of the medium. As cells grow, the offspring are released into the waste medium (called permeate, eluate or perfusate). By measuring the rate of cells released from the system and knowing the attached biofilm cell population the growth rate can be calculated. The growth rate can be controlled by changing the flow rate of medium. However, the membrane system is small and the corresponding samples that can be obtained for analysis are also small in volume and numbers of cells.

A more recent development is a modified version of the perfused biofilm fermenter termed the Sorbarod system (Hodgson *et al*, 1995). This has a biofilm surface area which is many times greater than in the membrane method and produces sufficient sample to allow biochemical determinations to be more conveniently made.

One important advantage of the Sorbarod perfusion model is that it is an open system which for a significant period of time (*c.* 3–4-day period) can be considered to be in steady-state when presented with a favorable physico-chemical environment (flow rate, media composition, temperature and pH). A second advantage is that the physical model itself is of a size that allows adequate samples to be taken for analysis without the sampling procedure disturbing the steady-state. With electrodes present it can be constantly monitored (Figure 1). Another important advantage with the *in vitro* perfusion model is that it has a

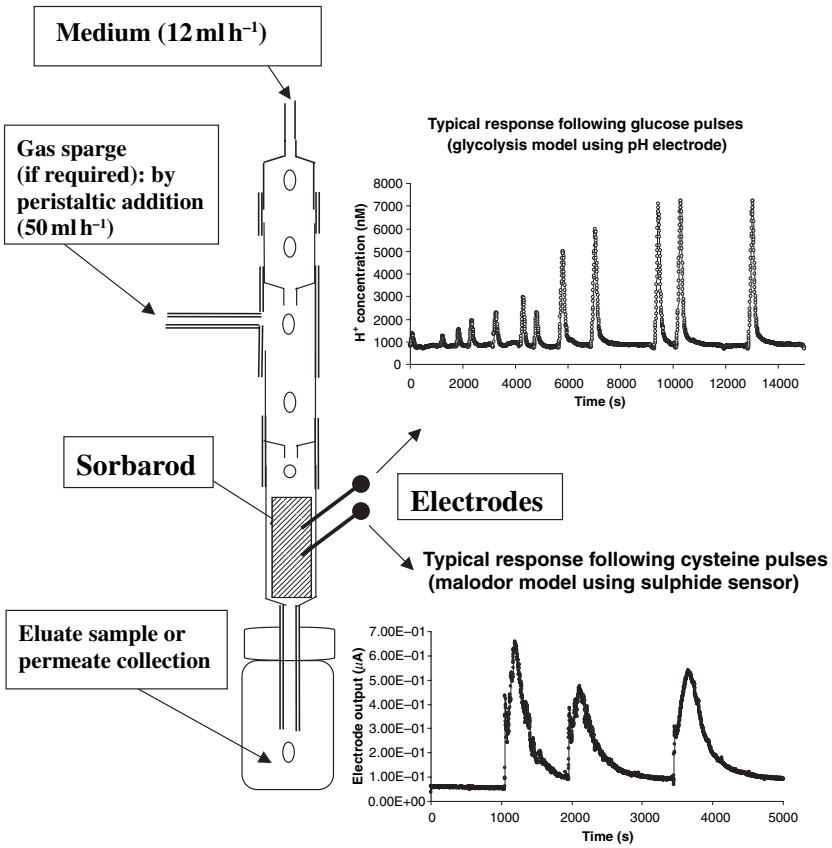

Figure 1 Sorbarod biofilm system

theoretical or mathematical counterpart giving a computer model of the same processes (Figures 2 and 3). The model can also be used for screening new types of antimicrobial compounds or anti-breath agents and comparing their efficacy against established agents against biofilm growth and VC/VSC production in the same model.

A perfusion biofilm incorporating a Sorbarod was employed to study complex mixed culture biofilms derived from tongue scrape inoculum (Greenman *et al*, 2002; Spencer *et al*, 2003). These biofilms were examined in terms of their ecological stability by sampling eluates and biofilms and recovering component species using conventional microbiological techniques (selective and non-selective recovery medium). The physiology of VSC production by the biofilm cells was studied using a cell suspension assay coupled to a halimeter. A quasi steady-state ($\mu = 0.014\ h^{-1}$) was achieved by 48 h and continued to the end of the experimental period (96 h). Comparison of biofilms with eluate and original inoculum showed their ecological profiles to be similar showing that the model maintained good ecological stability. Addition of S-substrates (cysteine, glutathione and methionine) to the growth medium resulted in samples with higher specific activity toward respective VSC substrates than controls thus demonstrating up-regulation (induction or de-repression) of VSC producing enzymes. Eluate and biofilm-derived samples were equivalent with regard to VSC-specific activities.

Some ideas for developing an overall 'bad breath model': 'what goes in must come out'

The most natural model is the natural ecosystem itself; the tongue biofilm *in situ*. The question remains, is it possible to model the overall process of oral malodor? The oral cavity is characterized by having three physical interactive phases: gas, liquid and solid. The gas/liquid mobile phases are highly dynamic and rapidly replaced. The replacement is described by the dilution rate, *D*, defined as the flow rate of gas or saliva divided by the volume of the oral cavity (for gas) or the residual salivary volume (for liquid).

Gas

The gas phase is characterized by rapid replacement and mixing. The dilution rate is about 40–80 h^{-1} although this is obviously reduced by breathing through the nose and shutting the mouth; it is increased by talking or (with blocked nose) breathing through the mouth.

Liquid

Mainly saliva, but some crevicular fluid (small volume but high concentration of S-containing substrates). The bulk fluid flows through the oral cavity at a dilution rate between 1.0 and 10.0 h^{-1}.

Solid

The solid phase consists of the tongue biofilm itself and the epithelial surfaces (which slowly exfoliate). The

A. Growth

Monod equations:

1. $\mu = \mu_{max} \frac{S}{K_s + S}$ where μ = specific growth rate, μ_{max} = maximum specific growth rate, S = concentration of growth limiting substrate, K_s = growth substrate saturation constant

2. $Y = \frac{dX}{dS}$ where Y = growth yield, X = biomass concentration

Rate of growth in sorbarod and in tongue biofilm:

$\mu = \frac{\text{rate of elution of cells}}{\text{biofilm population}}$ (flow rate × cfu ml^{-1} h^{-1}) / (cfu)

B. Enzyme reaction (biotransformation)

Michaelis–Menten equations:

1. $V = V_{max} \frac{S}{K_m + S}$ where V = initial rate of reaction, V_{max} = maximal enzyme velocity, S = concentration of rate limiting substrate, K_m = enzyme substrate saturation constant (Michaelis–Menten constant)

2. For an enzyme-immobilized bioreactor with well-mixed continuous flow:
Substrate flow in = substrate flow out + substrate consumption by reaction

$$QS_o = QS + k_r ES / K_m + S$$

where Q = volumetric flow rate of bulk fluid, S_0 = initial substrate concentration, S = final substrate concentration, k_r = rate of reaction (where $V_{max} = k_r E$), K_m = Michaelis–Menten constant, E = enzyme concentration

C. Inhibition of enzyme (e.g. by anti-odor mouthwash; non-competitive inhibition)

$V = V_{max} \frac{S}{\alpha(S + K_m)}$ where $\alpha = 1 + i/K_i$, K_i = enzyme inhibition constant, i = inhibitor concentration

D. Inhibition of growth of cells in biofilm (e.g. by antimicrobial agent)

$\mu = \mu_{max} \frac{S}{\alpha(S + K_s)}$ where $\alpha = 1 + i/K_i$, K_i = growth inhibition constant, i = inhibitor concentration

Figure 2 Some equations of use in modeling *in vitro* or *in situ* biofilm growth and oral malodor

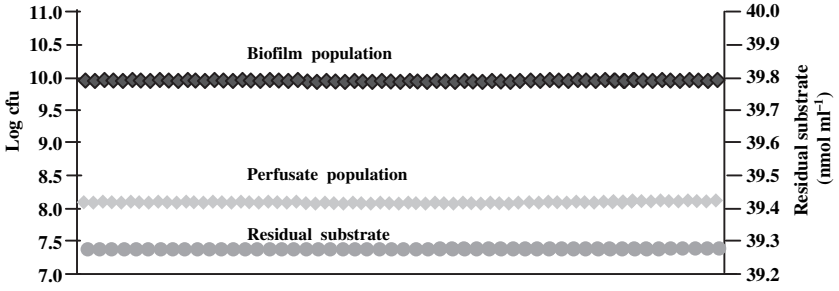

Figure 3 Computer (spreadsheet) model of the Sorbarod biofilm growth. Modeling was based on Monod's equation relating growth and substrate concentration. The perfusate flow rate chosen (24 ml h^{-1}) resulted in a growth rate of 0.018 h^{-1}

tongue architecture is complex with crypts and fissures supporting a high bacterial population. The biofilm is responsible for the biotransformations of immediate substrates to VC/VSC product and may also be responsible for increasing the amount of immediate substrates by enzymatic hydrolysis of protein or peptide secondary substrates.

In vitro studies suggest that the most important groups of organisms generating VCs and VSCs are anaerobes, particularly those GNA isolated from the tongue biofilm. Studies on human volunteers have revealed a correlation between subjects' oral malodor scores and log numbers of anaerobes, GNA and sulfide-producing organisms per unit area of tongue

surface (Hartley *et al*, 1996a). This relationship is also observed following the use of a metronidazole mouthrinse to selectively target the anaerobes (Hartley *et al*, 1999), where odor reduction and concomitant reduction of anaerobes and sulfide-producing organisms is highly significant ($P < 0.01$ at 24 h post-rinse). The mean number of bacteria on the tongue surface has been determined to be approximately 2×10^9 cm^{-2} (Hartley *et al*, 1996b). The macro-surface area of the tongue dorsum is approximately 30 cm^2. The resident tongue population is therefore approximately 6×10^{10} cells.

For studying cell growth we can consider the oral cavity as a continuous perfusion biofilm system. Saliva brings in nutrient substrates which allow the microbes to grow. Saliva also removes a portion of all components (eroded cells, low and high-molecular-weight products and residual substrates). As a first approximation it is reasonable to assume that the microbes grow in steady-state where their growth rate equals their removal rate. The removal rate is equal to the salivary total bacterial count per milliliter multiplied by the flow rate in milliliter per hour. According to Hartley *et al* (1999) the salivary bacterial count is approximately 2×10^8 per milliliter. Based on an unstimulated salivary flow rate of 12 ml h^{-1}, the total number of bacteria shed per hour equals 2.4×10^9. The production rate (growth rate, μ) is therefore: $2.4 \times 10^9 / 6.0 \times 10^{10} = 0.04$ h^{-1}.

Besides the addition of nutrients for bacterial growth and removal of bacterial cells shed from the surfaces, saliva also brings in sulfur-containing substrates (e.g. cysteine) which are biotransformed into VSC gases (e.g. H_2S). In steady-state, the production rate is equal to the removal rate (gas dilution rate). For studying VSC production, we can consider the oral cavity to be a continuous immobilized enzyme system (Figure 2). The amount of enzyme and its turnover is proportional to the amount of bacterial cells and their turnover and will be roughly constant.

The above considerations and equations shown in Figure 2 are the type that may be used to model bad breath by computer. To test out any computer model that may be developed, real data from real experiments in the human mouth will be needed. For example, cysteine rinses may be given to human volunteers and the kinetics of VSC production monitored using breath sensors.

Malodor on the breath is ultimately perceived by another person via the nose of the perceiver. This process can also be modeled. Although a kinetic model has yet to be proposed, an equilibrium model has now been published (Greenman *et al*, 2004).

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