Chapter 7
Discussion

From the literature review presented in Chapter 2, it is possible to make some general observations. For example, significant problems are frequently encountered in the independent replication of studies (Furia 1986, Gos 1997). Evidence is presented that the induction of effects may be multifactorial (Belyaev 2000). Some investigators interpret frequency-specific effects in terms of interaction with coherent excited modes of the Fröhlich type in the biological system. In addition, a number of confounding experimental factors have been identified that may cause the induction of frequency-specific effects, that are a consequence of physical, rather than biological factors (also see 2.7).

The approach adopted here for establishing the validity of non-thermal effects, which have been suggested both theoretically and experimentally, differs markedly from those used by previous experimenters. Prior experimental work has, by necessity, concentrated on very narrow bandwidths. In this study, significant effort has gone into developing a screening system with a high throughput potential. The methodology has been influenced by high-throughput systems developed in the pharmaceutical industry for the early-stage evaluation of candidate drugs. An initial concept based on the industry-standard multiple-sample-well format proved unsuitable hence the development of in-line segmented flow approach.

Frequency-specific effects have been reported to occupy a half-width as narrow as 1 MHz. Experimental protocols are designed so that the frequency steps between assays are of a sufficiently high resolution to detect the resonant biological effects. With the methodology described by Furia, (Furia et al. 1986) this would require at least 4 hours per iteration. To systematically screen even small portions of the mm-wave spectral

range at this resolution requires an inordinate amount of time using conventional assay approaches.

In certain respects, electromagnetic radiation is easier to screen than chemical compounds. For example, the problem of transport across the cell membrane does not exist. A significant challenge relates to the uniformity of power distribution or dosimetry within the sample under test. A further point: in drug discovery screening, to become a “candidate” a compound would normally need to demonstrate a high level of activity. However, small effects may be significant when considering mm-wave radiation because of the economics that free space transmission technologies offer in the irradiation of a large number of organisms at low intensity.

Attempts to replicate experiments independently often fail. One potential explanation is that the observations are a consequence of experimental artifact. The potential for artifact in experiments is high and this is in part due to the SARs that evolve superficially in lossy dielectrics (Ghandi 1983). Moreover, these are associated with a small but rapid temperature rise, which can cause convection phenomena (Khizhnyak & Ziskin 1996).

Another source of non-uniform heating relates to horn antennas and near- and far-field exposure regimes (Khizhnyak & Ziskin 1996). Simulation (see Chapters 5 and 6) was used to model the exposure cell. With this approach, spatial SAR could be determined as a function of frequency over the operational range of the cell. It is not practical in any exposure system to obtain a uniform SAR throughout the sample volume. However, simulation can be used to predict frequency-specific heating patterns that predispose to the induction of physical effects.

Favourable factors that tended towards uniform SAR included a sample thickness less than the skin depth, and a rounded geometry to remove edge effects. With a 0.5 mm internal bore the transit across the waveguide cavity gave a 4 μl sample volume. This is comparable to the

sample volumes used in ultra-high throughput systems. 0.5 mm is the minimum internal tube diameter that can be extruded in silicone. It also becomes increasingly difficult to operate peristaltic pumps at low flow-rates because of the uneven backpressure developed as each roller advances. As each segment progresses down the tube, some parabolic flow and diffusion effects will be operative in the sample itself. These are difficult to model numerically but will tend to enhance SAR uniformity rather than reduce it. A strategy used to minimize non-uniform heating effects is to re-circulate or stir the cell-culture (Furia et al. 1986).

Another important issue relates to experimental controls. A typical arrangement is illustrated by Gos (Gos et al 1997) and comprises two identical exposure chambers that are electrically isolated. In each single experiment, cells are taken from the same culture and are placed in either the exposure chamber or the sham exposure chamber. For growth rate, 2 standard deviations equate to ± 3.5 percent (Furia et al 1986). An inferior approach is to use a “historic” control where sham exposure and exposure are sequential rather than concurrent. This introduces significantly more biological variability (Gos et al 1997). This was confirmed in the start up of the continuous-culture system where significant variation in the growth rate and bioluminescence was observed (see Chapter 6).

Belyaev (Belyaev et al. 2000) emphasises the importance of biological parameters for experimental reproducibility. Interestingly, genome conformational change was observed in stationary phase cultures but not in exponential growth phase cultures (see Chapter 2 for additional details). The normal practice is to assay a growing culture (e.g., for a antibiotic) as this is the physiological phase where maximum rates of biosynthesis occur. Some biological phenomena are largely stationary-phase related, for example quorum sensing which is involved with intercellular communication (as in the bioluminescent bacterium *Vibrio fischeri* used in this study). If, as Belyaev suggests, mm-wave radiation can drive conformational change in the bacterial genome, then this would

represent a highly energy-efficient control mechanism. One can speculate that, on an energetics-based argument, this mechanism may be important in the stationary phase, but not in an exponentially growing culture.

The AVTD technique has not been well established in the literature. Moreover, it does not provide information about the functional significance of the genome conformation change. However, in connection with this, Belyaev (Belyaev et al 2000) put forward a hypothesis relating to the differential effects of circularly polarized mm-waves and that these may have a conformation-controlling role in B and Z-form DNA. In APPENDIX B, a statistical-mechanics approach based on the zipper model is used to identify regions with a propensity to form Z-DNA in a given dinucleotide sequence (Ho et al. 1986). This could be correlated with a genome-wide gene expression assay.

Effects on lower eukaryotes such as yeasts (see Chapter 2) represent an important, albeit highly contentious, area of research. One possibility is that mm-wave radiation may act as an input to the ultradian clock, which in combination with the mitotic oscillator gates intracellular processes (Lloyd 1991). Supporting the involvement of the ultradian clock are the studies performed by Golant (Golant et al. 1994) on the synchronisation of the yeast *Saccharomyces carlsbergensis*. Although the authors do not mention ultradian biology in their analysis, the 60 min. period in the control is consistent with independent studies on the ultradian biology of yeasts. The shift in periodicity from 60 min. to 80 min. with exposure at 46 GHz strongly indicates that the effect has a non-thermal basis. This is particularly the case because ultradian clocks 1) are temperature compensated 2) show periodicity changes in quantized steps.

Recent work has shown that the ultradian clock has a genetic basis and involves the *cdc2* control network (Lloyd 1991). Differential effects of mm-wave exposure may therefore be strain-specific. As the periodicity of the clock is different for each species, this forms a basis for selective

interaction in lower eukaryotes and mammalian cells, which could potentially have therapeutic application. Another target are limit cycles, a commonly observed type of oscillation in biological systems. These may collapse

Another interesting area of research relates to the induction of λ-prophage in *Escherichia coli*, which is one of the few effects that have been independently replicated (Webb 1979; Bannikov & Rozhkov 1980; Lukashevsky & Belyaev 1989). Prophage induction is generally related to factors that cause a transcription-inhibiting protein (in this case λ-repressor) to be released from a highly specific binding site base sequence at the start of the prophage genes. Release of the repressor activates a nearby RNA polymerase binding site, allowing synthesis of phage RNA and proteins and induction of the prophage. A critical step in this process is the removal of the hydrogen-bonded repressor protein from its DNA binding site, which can be related to a wide variety of cellular stresses but does not necessarily imply that mm-wave radiation is mutagenic as conformational changes in either the λ-repressor protein or the DNA binding site might lead to repressor desorption.

To achieve a flexible system that offered improvements in sensitivity, throughput rate, and physiological reproducibility the apparatus had to operate in a radically different way from previous batch- and plate-culture experimental designs. A continuous-culture device formed the basis of this new approach (Chapter 4). It allowed the sequential presentation of test samples in a consistent physiological state. By diluting the continuous-culture device product in a mixing chamber, it was possible to deliver different cell-densities into the exposure cell. For example, exponential and stationary phase cultures. In fact, the continuous-culture device allows long-term physiological reproducibility of the test organisms in virtually any growth state. The biology of the chemostat approach differs radically from previous batch culture exposure, allowing high uniformity of cell physiology in the population. This may be particularly important considering that the homeodynamics of biological systems may

be driven by controlled or deterministic chaos (Serra 1990) (Lloyd 1994) and this may represent a fundamental difficulty in replicating work. The continuous-culture system could operate autonomously over long-periods.

Disadvantages of the continuous-culture approach included the comparatively high cost of equipment and its increased complexity. Occasionally the experiments had to be stopped in an unscheduled way due to contamination with extraneous environmental organisms. The continuous-culture system (see Chapter 4) described was optimised for the generation of photobacterium. The control strategy was based on a dual set-point control that actuated medium flow in response to changes in bioluminescence and turbidity.

Growth rate and bioluminescence are both non-invasive and highly integrative reporters of biological effects. This is in contrast to the recent trend towards proteomics and genomics based assays. It argued here that simple integrative reporters are superior for preliminary screening purposes and offer a route to understanding the systematics of interactions in parameter space. The unit cost of assaying each bioluminescence segment is low whereas sophisticated genomic and proteomic analyses are expensive. These factors limit the scope of genomic and proteomic type assays to comparative studies involving spot frequencies. They are unsuitable for the high-throughput role. Likewise, higher organisms such as mice do not provide a good basis for exploring parameter space.

Experimental protocols relating to exposure duration vary widely. In the experimental studies performed by Furia (Furia 1986), the culture is re-circulated and intermittently exposed, whereas in later studies by Gos (Gos et al 1997) cells were continuously exposed for several hours. Transit time through the exposure cell is the principal limiting factor in the throughput rate of our in-line system. With an 8 s irradiation time, in excess of 200 assays hr^{-1} can be processed although it is considerably

slower with longer exposure times, which are more likely to be associated with biological effects. We justify comparatively short exposure times on the basis that our detection system is extremely sensitive. The 95 % confidence interval of the high-throughput system is approximately ± 0.1 % and this contrasts with non-continuous assays, which are of the order ± 5 %. It should also be noted that in a systematic screen, increased sensitivity reduces the number of assay points required.

An important point in comparison with the approach used by Gos (Gos et al 1997) for example, relates to noise and drift in the system and environmental parameters. In the high-throughput system all the detectors are positioned on the same panel and so, for example, a drift in enclosure temperature will be common to all channels. The high-throughput system also incorporates the concept of feedback. As described in Chapter 3, unexposed calibration segments (which are used are used to calibrate the confidence interval) are interleaved with those that are exposed. Feedback can be used to re-assay parameter space associated with any segment outside the pre-set confidence intervals. In this way, false-positives can be eliminated. Significant scope exits to introduce sophisticated elements to the control by for example using neural networks to control stimulus and feedback parameters.

The high-throughput system requires further development in a number of areas. The most significant limitation of the current system relates to the vector network analyser, which has a maximum operational frequency of 40 GHz. Extension of this frequency range to 110 GHz could be easily accomplished by purchasing the appropriate upgrade for the 8510C although it is expensive. For experimental work at higher frequencies in the mm-wave and sub-millimetre spectral range, vector network analysers are not commercially available. It is suggested here that an alternative technology, photomixing would be a more economic proposition, although the characteristics of the source, 1-2 MHz bandwidth are significantly broader than those used in the VNA. A potential scheme is outlined in appendices F and G. Polarization may be

an important parameter and could be investigated using an exposure cell capable of propagating circularly polarized radiation.

An important objective was to construct a system that was flexible. The continuous-culture device can handle other bacteria with genetically engineered luminescence reporters for a wide variety of biochemical activities. Similarly, tissue cultures of higher organisms with engineered luminescence reporters could also be substituted for the current bacterial systems.

In specific relation to the investigation of the Fröhlich condensate, one approach is to detect the emission of mm-wave photons from coherent excited modes using sensitive, passive radiometers. Another approach may be to gate a scanning mm-wave source with a passive radiometer and monitor the background emission. The concept is that, as energy is channelled to coherent excited modes, depletion will occur across a wide spectral range. The advantage is that it detects a change in Bose-Einstein statistics of energy rather than a biological effect and this occurs almost instantaneously. Sinitsyn (Sinitsyn et al. 2000) combines a radiometer and swept mm-wave stimulus. Self-tuning of the mm-wave stimulus was achieved using a drop in measured temperature.

Some early findings were reported using Raman spectroscopy although these are highly contentious. Lauck (Lauck et al. 1993) shows that the near Brillouin zone coincides with that used in Raman spectroscopy. An alternative approach to probing short wavelengths in the Brillouin zone is to use neutron-scattering spectroscopy. This technique is used to investigate phonon-modes in lattices, and could potentially be adapted for biological systems. The main problem relates to the biological damage caused by the neutrons, which may obliterate any experimental observations. Further investigation of the biological effects, at the required dose rates, may resolve this question.

Conclusions

After thirty years of research, no consensus has emerged relating to the existence of non-thermal effects of millimetre-wave radiation. Unlike other physical agents such as UV radiation, attempts to replicate millimetre-wave induced effect are often met with failure. It is suggested that this may be related to what has been termed *complex behaviour* in biological systems. This could involve self-sustained oscillators known as limit cycles. Biological systems exhibit *deterministic chaos* and this may account for the difficulty in the replication of studies. Recognition of these possibilities provided the basis for an instrument with a radically different mode of operation.

The high-throughput system utilized a continuous culture device. This device allows the presentation of biological samples to the exposure cell in a uniform physiological state. By adjusting the dilution factor in the mixing tank stage, samples in different phases of growth can be assayed. The exposure cell was simply constructed with a sample tube transecting the waveguide cavity that operated in the fundamental mode. The sample dosimetry within the exposure cell was characterized using vector network analysis and simulation. The simulation provided information on the uniformity of power deposition within the sample across the operational frequency range of the cell. Frequency-specific heating patterns that predispose to the induction of physical effect such as convection could be identified.

An important feature of the approach was its flexibility. Practically any culturable cell type is compatible with the operation of the instrument. The Photobacterium *Vibrio fischeri* acted as a highly integrated biosensor. The exposure cell operated in a “flow-through” mode where the flow was segmented to avoid convolution effects. Calibration and exposed segments were interleaved. The statistics of each calibration series were used to set a confidence interval, and any segment outside this interval

was a “candidate” biological effect. The parameter space associated with the candidate can be repeated. In addition, finer-grained steps inparameter space can be initiated. Potentially, feedback schemes can be much more complex and involve artificial intelligence and neural networks for example. The sensitivity of the system could exceed 0.1 %, which is a significant improvement over any prior system.

A short-term objective is to extend the operational frequency range of the instrument. This is necessary as the 8510c has a upper frequency of 40 GHz and this needs to be extended to 75 GHz to cover the frequency ranges historically associated with effects. Vector network analysers that operate at frequencies above 75 GHz are either unavailable or prohibitively expensive. An alternative technology, photomixing can offer a 600 GHz bandwidth in a single device but with a source half-width of around 2 MHz. The possibilities of conducting neutron scattering spectroscopy on living organisms and artificial cell-membrane systems should be considered.