CHAPTER-1

INTRODUCTION
Since the 18th century scientists have been intrigued by the interaction of electromagnetic fields (EMFs) and various life processes. Attention has been focused on EMFs in different frequency ranges, of which microwave frequency range forms an important part.

1.1 Microwaves

Microwaves are part of the electromagnetic spectrum and are considered to be that radiation ranging in frequency from 300 MHz to 300 GHz, which correspond to a wavelength range of 1m down to 1mm. This Study focuses upon an aspect of how living organisms, and humans, can be adversely affected by highly coherent electromagnetic fields of technological origin, in a way that is not entertained or addressed by existing Safety Guidelines – namely, through the possibility of non-thermal, frequency-specific influences of an informational nature and others.

The principle study focuses on those wave bands which held promise for use in instruments and appliances for general utility and popular applicability. Microwaves (MW) are used in many commercial devices, including television, microwave communication (mobile phone in particular), microwave oven, medical diathermy, radio navigation, long range radar and abundance of special equipment designed for specific uses.

Special attributes such as faster heating rate and greater penetration depth have made microwaves a unique tool for many industrial applications such as tempering, thawing, blanching, cooking, dehydration, sterilization and pasteurization. Even before the microwave oven was built, attempts to use microwaves to destroy micro-organisms had begun (Fleming 1944). The effectiveness of microwaves for sterilization has been well established by numerous studies over the previous decades (Latimer 1977, Sanborn 1982, Brown 1978, Goldblith 1967). The exact nature of the sterilization effect that whether it is solely due to thermal effects or to the 'microwave effect' has been a matter of controversy. Although there is a controversy about the mechanisms of microwave-induced death of micro-organisms, there is no doubt about the destructive effect of microwaves.
1.2 Interaction of microwaves with biological system

MW influence is observed on various microorganism taxons. Bactericidal impact of mm-waves, their influence on microorganism reproduction and different substances synthesis, proves mm-waves influence on animal cell vital functions needed to kill microorganisms.

This nonionising electromagnetic radiation is absorbed at molecular level and manifests as changes in vibrational energy of the molecules or heat. (Microwaves irradiating the community, Hidden hazards, Bantan Books publisher, Australia, 1991). One of the basic mechanisms underlying the interaction of microwaves with biological systems on the molecular level is the field-induced rotation of polar molecules and coherent excitation.

The interaction of radiofrequency (RF)/Microwaves with cell or tissue can be considered as the result of three processes:

1. Penetration by electromagnetic waves and their propagation into the living system.
2. Primary interaction of the waves with cell, tissue.
3. Possible secondary effects of the primary interaction.

The word interaction is important. It signals that end results depend not only on the action of the field but are influenced by the reaction of the living system. Living systems have a great capacity for compensating the effects induced by external influences, including electromagnetic sources. The integrity and functionality of all biological material is meticulously maintained by a complex network of biological “sensing” and “correcting” devices, which respond to changes in chemical and physical parameters in the local environment. The basis of most functional adaptation to cellular processes lies either at a) altered gene and protein expression, or b) altered protein activity, resulting from appropriate modulation by signal transduction mechanisms. The overall ability of cells to react in this manner is often collectively termed “stress response”. The true flexibility of the stress response is well illustrated by the wide variety of chemical and physical stimuli, which have been shown to elicit complex and functionally-co-coordinated alterations to gene and/or protein expression/function in cells. The biological logic embodied in the stress response lies in equipping the cell with a more robust phenotype.
by securing or enhancing major cellular housekeeping functions such as macromolecule (DNA, RNA and protein) synthesis and repair, and chemical energy supply. This adaptive “battle” against loss of function and initiation of cell death has several features that are noteworthy. Firstly, it is a characteristic of all biological systems, and is avidly preserved throughout evolution, from the simplest prokaryotes, throughout the eukaryotic kingdom. Secondly, although the stress stimulus may vary considerably, the molecular components of the stress response often contain commonalities. One source of a stress stimulus which has received less attention, but which is rapidly coming into focus from a biological and human health perspective, is that of electromagnetic radiation. Apart from more traditionally investigated aspects of this field involving exposures to ionizing radiation and UV radiation, exposure of biological material to radio frequency (RF) emissions is presently attracting attention, especially due to the use of microwaves in mobile telecommunications systems. Indeed, the scientific community is presently assessing if exposure of the human population to RF emissions and their incumbent magnetic fields is detrimental to health or not. Thus, there is a rapidly accumulating literature describing the potential stress response(s) of biological material to RF energy in particular. Therefore, it is the purpose of this treatise to give an overview of presently available data on RF exposure and its effect on prokaryotic system both in vitro and in vivo. At all times efforts will be made to compare and contrast the experiments in terms of a) the exposure, its physical characteristics etc., b) the dose-response characteristics (thresholds.), c) biological aspects of the response and d) methodological aspects of the detection of the molecular stress response not a central issue for this, essentially biochemical and biological review, a final summation will attempt to provide some suggestions to improve our knowledge in this area, as well as speculating on the potential impact of stress response data on the overall assessment of risk to the human population from exposure to RF emissions from mobile telephony (latest concern).
1.3. Concept supporting the experiments performed:

The present work is an attempt to support NONTHERMAL EFFECT OF MICROWAVE IRRADIATION ON BIOLOGIC SYSTEM, especially microorganisms. It will show the bactericidal action of microwaves in absence of temperature rise. The work is concerned with study of biological effect of magnetic field, as a component of non ionizing radiation on a unicellular system.

The frequency used for irradiation of microorganism is 2450MHz, Pathogenic microorganism especially Escherichia coli (gram negative) are chosen to be our experimental model for much reason, it is widely distributed in our environment such as soil, water, and air. E.coli is a member of normal intestinal flora of humans, it causes several diseases such as urinary tract infection, wound infection, traveler’s diarrhea. It reaches blood stream and causes sepsis and meningitis.

The study examine along with nonthermal lethal effect, the differences in the effect of microwaves on gram positive and gram negative bacteria. For this purpose gram positive bacteria Bacillus Subtilis cells have been taken. The genus Bacillus consists of a large number of diverse, rod-shaped Gram positive (or positive only in early stages of growth) bacteria that are motile by peritrichous flagella and are aerobic. B. subtilis is widely distributed throughout the environment, particularly in soil, air, and decomposing plant residue, B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active due to the various enzymes produced by members of the species. B. subtilis has been used for industrial production of proteases, amylases, antibiotics, and specialty chemicals.

The present experiment describes, how microwave radiation of 2450MHz frequency continuous wave radiation, at different exposure periods effect the cell activity and there survival or growth.
1.3.1 Growth

Growth in microorganisms is operationally defined as ability of irradiated organisms to multiply and form visible colonies, or increase in cell size e.g., coenocytic microorganisms have nuclear divisions that are not accompanied by cell divisions upon incubation on suitable growth medium. For this we will study the growth curve. The growth curve in most micro-organisms follows the exponential kinetics of simple target theory. Microbiologists usually study population growth rather than growth of individual cells.

<table>
<thead>
<tr>
<th>Method</th>
<th>Application</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct microscopic count</td>
<td>Enumeration of bacteria in milk or cellular vaccines</td>
<td>Cannot distinguish living from nonliving cells</td>
</tr>
<tr>
<td>Viable cell count (colony counts)</td>
<td>Enumeration of bacteria in milk, foods, soil, water, laboratory cultures, etc.</td>
<td>Very sensitive if plating conditions are optimal</td>
</tr>
<tr>
<td>Turbidity measurement</td>
<td>Estimations of large numbers of bacteria in clear liquid media and broths</td>
<td>Fast and nondestructive, but cannot detect cell densities less than $10^7$ cells per ml</td>
</tr>
<tr>
<td>Measurement of total nitrogen or protein</td>
<td>Measurement of total cell yield from very dense cultures</td>
<td>only practical application is in the research laboratory</td>
</tr>
<tr>
<td>Measurement of biochemical activity e.g. O$_2$ uptake CO$_2$ production, ATP production, etc.</td>
<td>Microbiological assays</td>
<td>Requires a fixed standard to relate chemical activity to cell mass and/or cell numbers</td>
</tr>
<tr>
<td>Measurement of dry weight or wet weight of cells or volume of cells after centrifugation</td>
<td>Measurement of total cell yield in cultures</td>
<td>probably more sensitive than total nitrogen or total protein measurements</td>
</tr>
</tbody>
</table>

Table 1: Some Methods used to measure bacterial growth
1.3.1.1. The bacterial growth curve

In the laboratory, under favorable conditions, a growing bacterial population doubles at regular intervals. Growth is by geometric progression: $1, 2, 4, 8, \text{ etc. or } 2^0, 2^1, 2^2, 2^3 \ldots \ldots 2^n$ (where $n = \text{ the number of generations}$). This is called exponential growth. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in Nature.

When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a typical bacterial growth curve (Figure 1 below).

![Figure 1. The typical bacterial growth curve.](image)

When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly by the process of binary fission (exponential phase). When their growth becomes limited, the cells stop dividing (stationary phase), until eventually they show loss of viability (death phase). Note the parameters of the x and y axes. Growth is expressed as change in the number viable cells vs. time. Generation times are calculated during the exponential phase of growth. Time measurements are in hours for bacteria with short generation times.
1.3.1.2. Four characteristic phases of the growth cycle are recognized

1. Lag Phase. Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity.

The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum's; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

2. Exponential (log) Phase. The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as generation time, also the doubling time of the bacterial population. Generation time (G) is defined as the time (t) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time (below) derive.

3. Stationary Phase. Exponential growth cannot be continued forever in a batch culture (e.g. a closed system such as a test tube or flask). Population growth is limited by one of three factors: 1. exhaustion of available nutrients; 2. accumulation of inhibitory metabolites or end products; 3. exhaustion of space, in this case called a lack of "biological space".

During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce secondary metabolites, such as antibiotics, do so during the stationary phase of the growth cycle.
(Secondary metabolites are defined as metabolites produced after the active stage of growth). It is during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process.

4. Death Phase. If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. (Note, if counting by turbidimetric measurements or microscopic counts, the death phase cannot be observed.). During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

1.3.1.3. Generation Time

As mentioned above, bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH, etc.), define the bacterium's generation time. Generation times for bacteria vary from about 12 minutes to 24 hours or more. The generation time for E. coli in the laboratory is 15-20 minutes, but in the intestinal tract, the coliform's generation time is estimated to be 12-24 hours. For most known bacteria that can be cultured, generation times range from about 15 minutes to 1 hour. Symbionts such as Rhizobium tend to have longer generation times. Many lithotrophs, such as the nitrifying bacteria, also have long generation times. Some bacteria that are pathogens, such as Mycobacterium tuberculosis and Treponema palladium, have especially long generation times, and this is thought to be an advantage in their virulence.

Calculation of Generation Time

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, and 8 cells in the third generation, and so on.
1.3.1.5. The Mathematics of Growth

- Generation (doubling) time
  - Time required for the population to double in size
- mean growth rate constant
  - Number of generations per unit time
  - Usually expressed as generations per hour

\[ N = N_0 2^n \]

\[ n = \log(N) - \log(N_0) \times 0.301 \]

N = final cell number

n = number of generations

g = \frac{t}{n} \quad g = \text{generation time}

N_0 = \text{initial cell number} \quad t = \text{time in hours or minutes}

So with above method we would determine change in GROWTH PATTERN of bacterial population after microwave exposure.

1.3.2. The Bradford assay

Supporting this we will quantify change in total protein content after microwave exposure with the help of "BRADFORD PROTEIN ASSAY TECHNIQUE".
1.3.2.1. Considerations for use

The Bradford assay is very fast and uses about the small amount of sample. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assessing protein concentrations for gel electrophoresis. It is sensitive to about 5 to 200 micrograms protein, depending on the dye quality.

1.3.2.2. Chemistry of Coomassie-based Protein Assays

In the acidic environment of the reagent, protein binds to the Coomassie dye. This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465 nm) to the blue form of the dye (absorbance maximum at 610 nm). Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change, as explained in Figure 2 below.

Development of color in Coomassie dye-based protein assays has been associated with the presence of certain basic amino acids (primarily arginine, lysine and histidine) in the protein. Van der Waals forces and hydrophobic interactions also participate in the binding of the dye by protein. The number of Coomassie dye ligands bound to each protein molecule is approximately proportional to the number of positive charges found
on the protein. Free amino acids, peptides and low molecular weight proteins do not produce color with coomassie dye reagents. In general, the mass of a peptide or protein must be at least 3,000 daltons to be assayed with this reagent. The difference between the two forms of the dye is greatest at 595 nm, so that is the optimal wavelength to measure the blue color from the Coomassie dye-protein complex. If desired, the blue color can be measured at any wavelength between 575 nm and 615 nm. At the two extremes (575 nm and 615 nm) there is a loss of about 10% in the measured amount of color (absorbance) compared to that obtained at 595 nm.

1.3.3 Variation in pH Affecting Bacterial Growth Curve: It has also been checked that whether change in pH have any influence on growth pattern of microwave exposed bacterial population. pH is a measure of hydrogen ion activity of solution and is defined as the negative logarithm of hydrogen ion concentration. It is not surprising that pH effect bacterial growth, each species have definite pH range and definite pH optimum, but there is limit to their tolerance drastic variation in pH can harm micro-organisms by disrupting the plasma membrane or inhibiting the activity of enzymes and membrane transport protein. Changes in pH also might alter the ionization of nutrients and thus reduce their availability to the organisms. We will determine the combine effect of pH and microwave radiation on growth pattern of bacteria. We have to study pH gradient instability created due to chemicals, or any change in BOD and COD, so that we can more efficiently treat waste water with the help of microwaves.

1.3.4 The Exposed Bacterial Cells Were Tested for Their In Vitro Susceptibility to Various Antibiotics. For this process, Agar diffusion method have been used. The principle used here is that antibiotic will diffuse from a paper disc or small cylinder into an agar medium that contains test organism. Inhibition is observed as a failure of organism to grow in the region of antibiotic. The appearance of zone of inhibition surrounding the disc is indicative of sensitivity. By comparing the diameter of zones of control and treated samples we have determined the organism is susceptible to what extent to antibiotics.
1.4 Microwaves and prokaryotic system

Importance of this work lays in the fact that *E. coli* as a microorganism is a unit cell behaving as a complete alive biological system.

With microbial cells and viruses, it is almost always possible to work with what are, effectively, "haploid" cells so that recessives are immediately expressed. Further, the generation times are of the order of tens of minutes or hours instead of weeks, months or longer, and it is possible to follow literally billions of progeny.

Microwave destruction of many bacteria has been reported including *Bacillus cereus, Campylobacter jejuni, Clostridium perfringens, E. coli, Enterococcus, Listeria, monocytogenes, Staphylococcus aureus, Salmonella enteridis, Salmonella sofia, Proteus mirabilis* and *Pseudomonas aeruginosa* (Chipley 1980; Knutson et al. 1987; Rosenberg and Bogl 1987; Heddleson and Doores 1994; Papadopoulou et al. 1995; Datta and Davidson 2000). No pathogen has been reported to be microwave resistant (Datta and Davidson 2000).