

# Extremely High Frequency Electromagnetic Fields at Low Power Density Do Not Affect the Division of Exponential Phase *Saccharomyces cerevisiae* Cells

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Exponentially growing cells of the yeast *Saccharomyces cerevisiae* were exposed to electromagnetic fields in the frequency range from 41.682 GHz to 41.710 GHz in 2 MHz increments at low power densities ( $0.5 \mu\text{W}/\text{cm}^2$  and  $50 \mu\text{W}/\text{cm}^2$ ) to observe possible nonthermal effects on the division of this microorganism. The electronic setup was carefully designed and tested to allow precise determination and stability of the electromagnetic field parameters as well as to minimize possible effects of external sources. Two identical test chambers were constructed in one exposure system to perform concurrent control and test experiments at every frequency step under well-controlled exposure conditions. Division of cells was assessed via time-lapse photography. Control experiments showed that the cells were dividing at submaximal rates, ensuring the possibility of observing either an increase or a decrease of the division rate. The data from several independent series of exposure experiments and from control experiments show no consistently significant differences between exposed and unexposed cells. This is in contrast to previous studies claiming nonthermal effects of electromagnetic fields in this frequency range on the division of *S. cerevisiae* cells. Possible reasons for this difference are discussed. *Bioelectromagnetics* 18:142–155, 1997. © 1997 Wiley-Liss, Inc.

**Key words:** cell cycle; electromagnetic field; nonthermal effects; yeast; extremely high frequency

## INTRODUCTION

Thermal effects of electromagnetic fields in the microwave range are well established and are common knowledge in virtually every household in industrialized countries. Nonthermal effects of extremely high frequency electromagnetic fields (EHF-EMF), however, are less well established. An intense debate has arisen regarding whether the described biological effects of electromagnetic fields in general, and of EHF-EMF at low power densities in particular, are real or not [Bise, 1978; Webb, 1979; Stone, 1992; Davis et al., 1993; Ahlbom and Feychting, 1993; Marani and Feirabend, 1993; Adey, 1993; Blank, 1993; Macklis, 1993; Bassett, 1993; Ellingsrud and Johnsson, 1993]. The increasing use of electromagnetic fields in telecommunications and for other purposes affecting everyday life makes settling this scientific debate an important issue. At a stage of conflicting experimental observations, a simple experimental system with a

model organism is called for, so that many experiments can be performed easily under biologically and technically well-controlled conditions. Microorganisms, and in particular the yeast *Saccharomyces cerevisiae*, provide such a model system. It has been shown that yeasts are representative of eukaryotes, including human cells, in many aspects of fundamental cellular processes [Botstein and Fink, 1988].

Among the reports on nonthermal biological effects of EHF-EMF [Webb and Dodds, 1968; Parker et al., 1988; Verma and Dutta, 1993; Sarkar et al., 1994; and see Furia et al., 1986, and Grundler et al., 1988, for references], the work with the yeast *S. cerevisiae* stands out, because experimental details have been reported and continuity of the research is discernible

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[Grundler et al., 1977, 1988; Grundler and Keilmann, 1978, 1989; Grundler, 1989, 1992]. With a basis in earlier work [Devyatkov et al., 1974; cited and reviewed in Grundler et al., 1988], Grundler and colleagues developed two independent experimental protocols. They published data suggesting nonthermal effects of EHF-EMF on the division of *S. cerevisiae*. In the first protocol, *S. cerevisiae* cell suspensions were exposed to EHF-EMF with frequencies in the range from 41.412 GHz to 41.898 GHz. The growth rates were assessed by turbidity measurements. Frequency-dependent changes in growth were reported, with enhancement up to 115% and inhibition down to 71% in the range from 41.6 GHz to 41.8 GHz [Grundler et al., 1977; Grundler and Keilmann, 1978]. An independent reproduction of this experiment was attempted but failed [Furia et al., 1986]. In a second, improved, experimental setup, cells were exposed to EHF-EMF on agar slabs [Grundler and Abmayr, 1983; Grundler et al., 1988; Grundler, 1989; Grundler and Keilmann, 1989]. Frequency-dependent changes of the division rate with enhancement up to 108% and inhibition down to 74% or 62% were observed in the range of 41.690 GHz to 41.705 GHz [Grundler, 1992; Grundler et al., 1992].

## EXPERIMENTAL DESIGN

The objective of this study was the confirmation of observations on nonthermal effects of EHF-EMF on the division rate of *S. cerevisiae* made by Grundler and colleagues [Grundler et al., 1988; Grundler, 1989, 1982; Grundler and Keilmann, 1989] as a basis for mechanistic studies on the interaction of electromagnetic fields with cellular components. The overall design is similar to the second protocol of Grundler [see Grundler and Abmayr, 1983, and Grundler et al., 1988, for details]. *S. cerevisiae* cells were exposed on agar surfaces, and division was recorded via time-lapse photography. For the purpose of this paper and in concordance with the field of cell cycle research, division is defined as the increase in cell body number, whereas growth is defined as mass increase of an individual cell or bud (see also the legend to Fig. 1). The term "growth" is also applicable to microbial populations, in which it is monitored by optical means. The EHF-EMF parameters of Grundler and colleagues were closely followed. We added the important feature of careful shielding from external sources by the design of the exposure chambers. Instead of cell synchronization [Grundler and Abmayr, 1983; Grundler et al., 1988], we decided to use undisturbed cells growing exponentially. Such a population contains cells in all stages of the mitotic cell cycle (G1, S, G2, M), as shown in Figure 1. For data acquisition, only cells without a bud

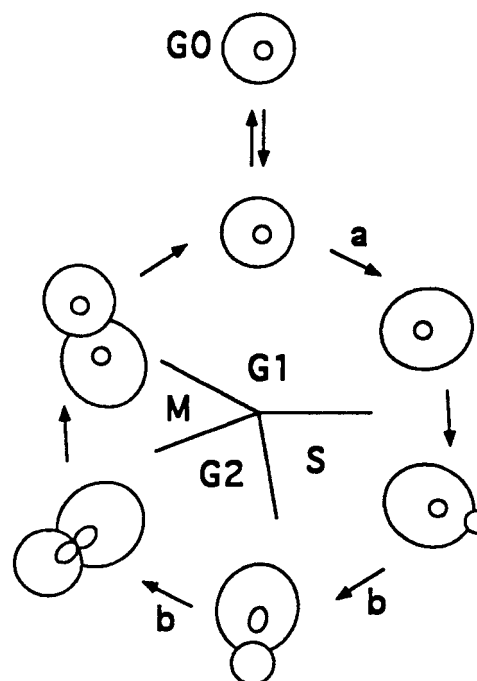


Fig. 1. Cell cycle of the yeast *Saccharomyces cerevisiae*. Exponentially growing *S. cerevisiae* cultures contain cells representing all stages of the cell division cycle (G1, S, G2, and M). Single cells in exponentially dividing cultures represent cells in the G1 phase of the mitotic cell cycle. *S. cerevisiae* divides by budding, and the emergence of the bud indicates the beginning of S phase. Cells with large buds include G2- and M-phase cells. Single cells in resting cultures are labeled G0 to indicate the clear physiological difference between actively cycling and resting cells. This difference cannot be easily assessed by simple visual inspection. The figure shows schematically the behavior of the cells and the nucleus during the mitotic cell cycle. Division occurs once per cycle. Growth occurs in G1-phase cells to achieve a critical mass before initiation of S phase (labeled a) and in buds during S phase and G2 phase to achieve a critical bud size before cell division and separation (labeled b; for a review, see Forsburg and Nurse [1991]; see also text).

and cells with a small bud were used. They precisely correspond to G1-phase and S-phase cells, respectively (see Fig. 1). Therefore, we could analyze the possible effects of EHF-EMF on cells in well-defined division-cycle states, without disturbing the cells by synchronization procedures. Disturbances are undoubtedly caused by any procedure of synchronization [Forsburg and Nurse, 1991].

An important feature of the present design is the concurrent control with unexposed cells. For every single experiment, cells of the same culture were used simultaneously in the exposed and unexposed chambers. Comparisons for unraveling a possible field effect were restricted to such concurrent pairs. This made possible the direct statistical comparison of data from exposed and unexposed cells.

Herein we report our attempt to obtain independent confirmation of a nonthermal EHF-EMF effect on the division rate of *S. cerevisiae*. We have used a setup similar to the second experimental protocol described by Grundler (see above). The technical parameters of the previous protocol were closely followed, but the experimental system was independent. Using concurrent controls of unexposed cells for every single exposure experiment, we were unable to detect consistently significant differences between exposed and unexposed cells at any EHF-EMF frequency tested. Possible reasons for this discrepancy from the previous studies of Grundler and collaborators are discussed.

## MATERIALS AND METHODS

### Strain and Media

For all experiments, the diploid *S. cerevisiae* strain WDHY376 (*a/α URA3/ura3-52 leu2Δ1/LEU2*) was used. It derived from strains FY2 and FY69, originally supplied by Dr. F. Winston (Harvard Medical School, Boston, MA). They are isogenic derivatives in the S288c background, the most commonly used laboratory background for *S. cerevisiae* [see Sherman, 1991]. The strain was preserved in 15% glycerol at  $-70^{\circ}\text{C}$  as described by Sherman [1991]. Rich medium (YPD; 2% Bacto-peptone, 1% Bacto-yeast extract, 2% dextrose, and optionally 2% agar) and medium for the selection of respiration-proficient cells (YPG; 2% Bacto-peptone, 1% Bacto-yeast extract, 3% glycerol, and optionally 2% agar) were prepared as described by Sherman [1991].

### Exposure Chamber Design

The design of a double-chamber exposure system extends the original design of Grundler [1992]. The system consists of a solid stainless steel block containing two exposure chambers each fed by a long horn antenna (Fig. 2). The two petri dishes were placed upside down on top of the exposure chamber. During exposure, a stainless steel cover was placed over the exposure chambers, providing electromagnetic and environmental sealing. The mechanical construction was done by a combination of machining and spark gap milling. The block and cover have inside bores for water circulation to allow temperature control. In the block, five temperature probes (PT 100) close to the exposure chambers were used for temperature recording and feedback for the temperature control circuit. The temperature was stabilized by a circulating water bath (Julabo; F10 + VC/3). Due to the mass of the metallic block, the short-term stability of the temperature was high. Temperature variations over

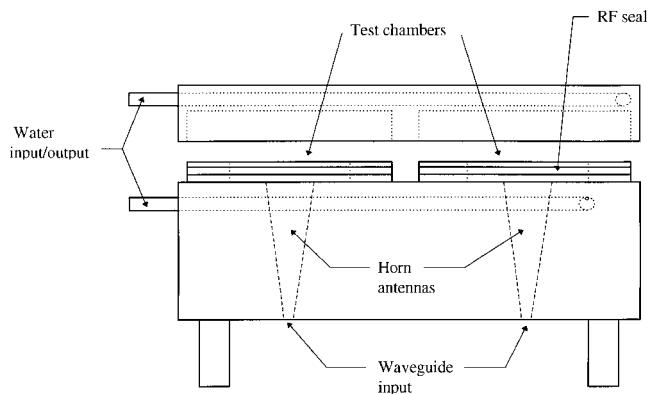


Fig. 2. Exposure system containing two identical test chambers for 40–45 GHz. In the upper part and the cover, the water tubing for temperature stabilization is shown with dotted lines. At the input waveguide of the horn antennas, pressurized dry air was fed into the horn antenna. A controlled air-flow was forced by using defined air leaks (0.1-mm-diameter glass capillary) close to the opening of the antenna (not shown). To prevent the entrance of contaminants or humidity, a mylar membrane was used to cover the antenna opening.

24 h were well below  $0.1^{\circ}\text{C}$  for the two chambers. The block was preheated for 24 h to reach temperature stability before experiments. The exposure system is shown in Figure 3 with the cover removed.

### Cell Carrier Design

The cells were placed on standard agar medium (YPD) in petri dishes (diameter 8.6 cm). The dielectric properties of agar were such that even for thin layers the attenuation of electromagnetic fields at the frequencies used for this experiment (40–43 GHz) was high. This implies that the exposure of the cells had to be performed from the side on which the cells were located. The cells were photographed before and after exposure on a micromanipulator (MSM System; Singer Instruments, Somerset, England). To permit repeated semiautomated location of the same cells, the petri dishes had a handle, so that they could be placed always in the same position either in the exposure chamber or in the system for microscopy and photography. The accuracy of the mechanical position of the petri dish was found to be better than  $\pm 0.01$  mm.

### RF Design

The design fulfills two main purposes: 1) control of RF energy distribution over the whole observation surface of the petri dishes and 2) decoupling of the electromagnetic fields between the chambers for control and exposed cells. To facilitate the use of the exposure system, no electrical tuning of the RF circuits (to compensate for mismatches) was required. This was

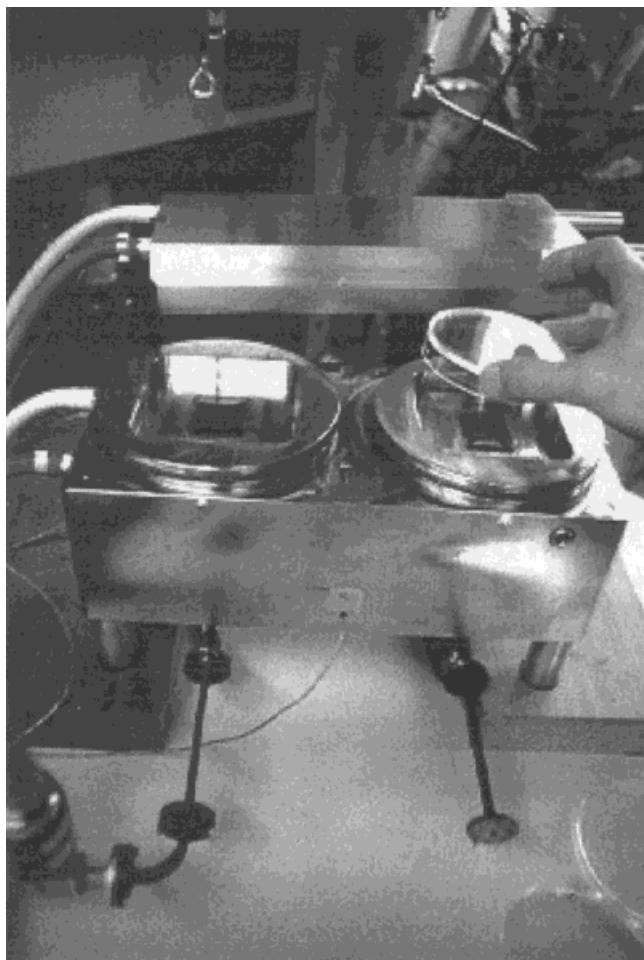


Fig. 3. Block with cover removed showing the two identical exposure chambers with the petri dishes. The petri dishes with the cells were placed upside down. The connecting leads of some of the temperature probes can be seen, as well as the water supply tubing for temperature stabilization. The cover was placed on a support behind the exposure chamber.

achieved by optimization of the thickness of the agar layer to result in a uniform reflection factor over the frequency band of interest, based on measurements obtained with an automatic network analyzer. For the exposure system used, optimal conditions were found with an agar layer thickness of  $0.86 \pm 0.05$  mm. For these particular experiments, the precise amount of absorbed RF energy was less important, because all experiments were made at levels below the limits for thermal effects. The amount of energy deposition was calculated for the highest power levels used in the experimental series. At the maximum power density used ( $50 \mu\text{W}/\text{cm}^2$ ), the specific absorption rate (SAR) was between 0.8 and 1.33 W/kg.

The petri dishes were placed at the aperture ( $3 \times 4$  cm) of the horn antennas. The cells were at the sur-

face of an agar substrate at a distance of 12.6 mm from the aperture. To obtain a uniform power flux distribution, a reduced surface of  $2 \times 2$  cm in the center of the aperture was used for the observation of the cells. Over the observation surface, the power flux variation was within  $\pm 3$  dB as measured with a small dipole antenna. The connecting waveguides and the horn antennas were pressurized with dry air to prevent moisture condensation due to the temperature difference between the heated metallic block and the room temperature. This was achieved by controlled air leakage from a glass capillary in the horn antenna. To prevent air flow into the exposure chamber, the horn antennas were sealed with a mylar foil of 0.1 mm thickness.

The RF signal decoupling between the two exposure chambers in the frequency range of 32–75 GHz was measured to be greater than 100 dB. Signals below the cutoff frequency of the connecting waveguide (IEC R 500) are attenuated inherently and need not be considered. The external RF circuit was designed for maximum signal stability in frequency and amplitude. Figure 4 shows the RF circuit. In the course of the experiments, two different RF-generating devices were used. Both sources had the same performance for the important RF characteristics summarized in Table 1. For further characterization of the RF signal parameters, the spectra close to the carrier frequency were measured (Fig. 5). No noticeable low-frequency (LF) modulation was observed.

#### Low-Frequency Design

The exposure system was designed for full control over extremely low frequency (ELF) fields as well. The magnetic fields were not shielded at all, to achieve the same ambient exposure for control and experimental cells. This was possible due to the material properties of the stainless steel block, which was fully trans-

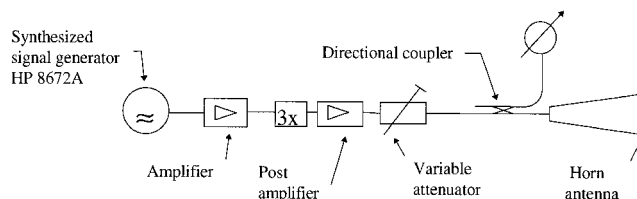


Fig. 4. RF-generating circuit for frequencies of 40–45 GHz. Initially, a frequency tripler with a transistorized postamplifier was used to generate power densities of  $50 \mu\text{W}/\text{cm}^2$  at the location of the cells. Experiments with lower power densities were performed with a synthesized generator HP 8340A, followed by a frequency doubler without pre- and postamplifiers. The electrical performances of both signal-generating systems were comparable.

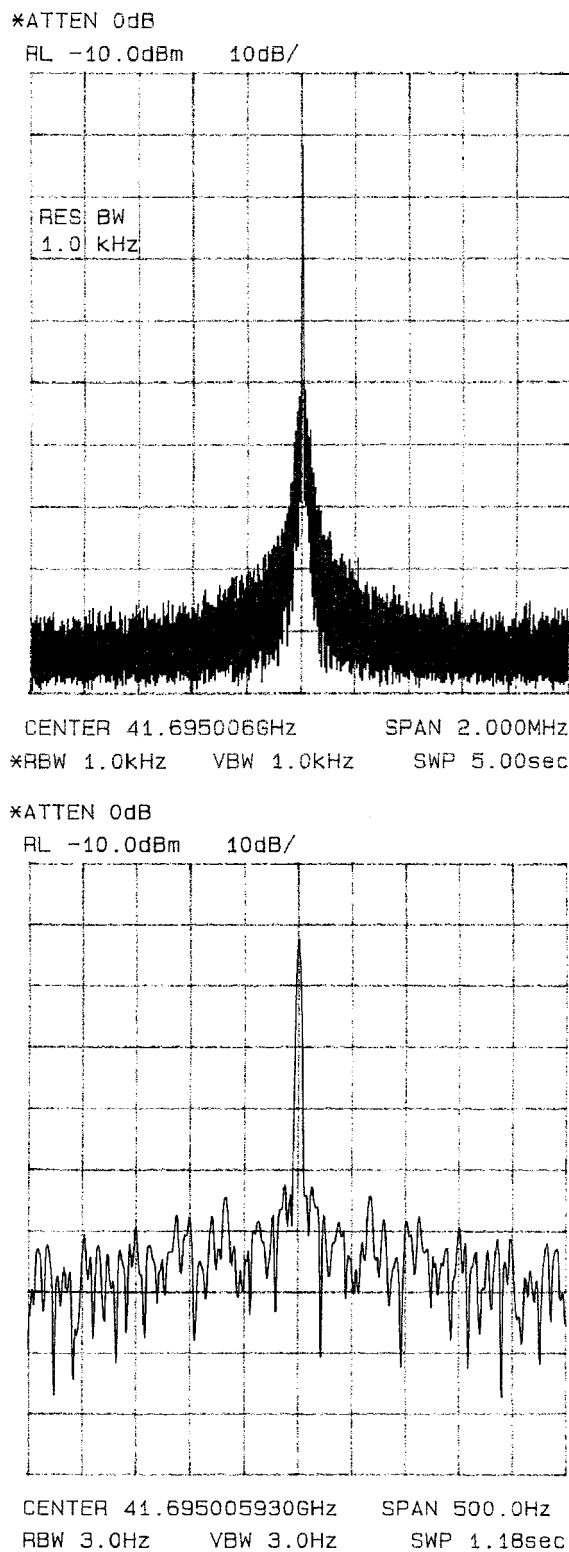


Fig. 5. Example of the output spectra close to the carrier frequency (41.695006 GHz) over a frequency span of 2 MHz and 500 Hz. The vertical scale is 10 dB per division, and the horizontal scale is 200 kHz and 50 Hz per division.

TABLE 1. Radiofrequency (RF) Field Parameters

Center frequency accuracy	$6 \times 10^{-6}$
Center frequency stability	$<1 \times 10^{-7}/\text{day}$
Frequency resolution	9 kHz
Harmonics and subharmonics	-10 dB below carrier or better
Amplitude precision	$\pm 0.3$ dB

parent to ELF magnetic fields as verified by using a miniature (4.7 mm diameter) magnetic field-sensing coil inside and outside the block. At 400 Hz, the external magnetic fields were attenuated by 10 dB. The attenuation vs. frequency of the magnetic field was found to increase by at least 6 dB per octave. Before the tests, the Earth's magnetic field and the ambient ELF magnetic fields had been measured and recorded over extended periods. During some experiments, the ambient magnetic fields were monitored as well. Spectral analysis of the ambient magnetic fields revealed frequency components of 16.67 Hz (originating from the electric railway system) and harmonics of 50 Hz. These spectral components were found to be at most 6 dB below the 50 Hz signal. According to Grundler's protocol (personal communication), the block was adjusted so that the horizontal component of the Earth's magnetic field was parallel to the RF magnetic field and perpendicular to the RF electric field. The ELF magnetic field data are summarized in Table 2. For external low-frequency and high-frequency electric fields, the exposure chamber acted as a Faraday enclosure with high attenuation (better than 60 dB from 0 Hz to 1 GHz, above 1 GHz >100 dB).

During a period of 3 years, the exposure system was used for cell growth experiments and has proved to be robust and stable. No degradation of the electrical parameters has been found. Control experiments at temperatures in the range of 28–34 °C have shown that the differences for the growth of the *S. cerevisiae* cells after 6 h were well within the normal biological variation.

### Experimental Protocol

For each experiment, WDHY376 was taken from frozen stocks and incubated on YPG plates at 30 °C for 2–3 days to select for respiration proficiency and against petite cells. Petites have mutations in their mitochondrial genome resulting in less efficient use of the carbon source and hence in slower growth [Sherman, 1991]. This step efficiently eliminated this problem. Cell material was then streaked to obtain single colonies on YPD and incubated at 30 °C. A single colony was used to inoculate a preculture with liquid YPD, which was then incubated on a wheel to stationary

**TABLE 2. Extremely Low Frequency (ELF) Field Parameters**

Static magnetic field	44.5 ± 1.5 mT
Background ELF magnetic field strength	120 ± 30 nT
Harmonics and subharmonics of line frequency (50 Hz)	-6 dB below carrier or better

phase at 30 °C. The wheel delivered an electromagnetic field of 600 nT at 50 Hz at the position of the cells. After overnight incubation, the cells were diluted into a second preculture for growth at 30 °C overnight to a titer of  $0.5-3 \times 10^7$  cells/ml. Choosing this titer ensured that the cells were in the exponential phase of the growth curve. At this stage, the same numbers of exponentially growing cells ( $2.5-15 \times 10^4$ , depending on the culture) were spread on a thin layer of YPD in two petri dishes. The same number of cells of one culture was used for the unexposed control chamber and for the chamber with field exposure. The vortex used to mix the cells had an electromagnetic field of 20  $\mu$ T at 50 Hz.

Both petri dishes were put into a micromanipulation apparatus (Singer Instruments) with a computer-controlled table and a microscope connected to a video camera with thermoprinter output. A predetermined grid pattern of 100 points was followed within an area of 400 mm<sup>2</sup> at the center of the antenna in the exposure chamber. Pictures were taken at every point ( $t_0$  pictures). During this procedure, the cells were in a 500–800 nT 50 Hz field for maximally 15 min. Both plates were then put into the exposure chambers for 5.5 h, one with a specified electromagnetic field, the other without a field. The chamber with field exposure could be chosen freely. No systematic difference between cells grown in the two chambers was apparent (data not shown). After this incubation, the petri dishes were stored on ice to prevent further growth, until pictures ( $t_1$  pictures) were taken again in the same predetermined grid pattern as before.

#### Data Acquisition and Analysis, Statistical Tests

Exponentially growing cells of *S. cerevisiae* can be distinguished easily by microscopy. G1-phase and S-phase cells are defined with high precision as unbudded cells and cells with a small bud, respectively (Fig. 1). The distinction between G2-phase and M-phase cells is not possible without further criteria. We recorded all cells for data acquisition and analyzed all cells satisfying a predetermined set of criteria to avoid a subjective bias in choosing the cells to be analyzed. 1) We analyzed all cells with no bud (G1) or with a small bud (S) that were present in  $t_0$  pictures. 2) The cells had to be sufficiently isolated so that the microcolonies in the  $t_1$  pictures were not overlapping. 3) Cells

not giving rise to progeny, presumably dead cells, which always occur in cultures, were recorded but not entered for data analysis.

The occurrence and the amount of nondividing cells showed no correlation with field exposure (data not shown). Between 14 and 103 cells, with an average of 45 cells, were analyzed per individual data point. A data point represents either G1-phase or S-phase cells, with or without field exposure. For a given frequency, a total of 77–349 cells were analyzed per experiment, with an average of 179 cells. The number of cell bodies deriving from a single cell were counted in  $t_1$  pictures, and this is a direct measure for the division rate. A cell body is a cell or a bud; thus, a budded cell is counted as two cell bodies (see legend to Fig. 1). This procedure increases the sensitivity of the experiment and avoids a subjective bias in the counting process. For three experiments, an additional count of the cell body numbers in all pictures was performed by an independent person, and essentially the same results were obtained (data not shown).

The data for G1-phase and S-phase cells were treated separately throughout data acquisition and statistical analysis. Therefore, for every experiment (i.e., with a given field frequency), we obtained two graphs, each with two distributions of the relative frequencies of cell body numbers deriving from the original cells. One graph refers to G1-phase cells (with and without field exposure), the other to S-phase cells (with and without field exposure). Examples of a control experiment without field exposure and of an experiment with field exposure are shown in Figure 6. The data were then treated with statistical methods. First, means were calculated from the individual distributions and compared between cells concurrently exposed and unexposed. An example is shown in Figure 8. The differences between these means were statistically analyzed using the z test [Sokal and Rohlf, 1981]. As in all experiments the number of observations (n, number of cells at  $t_0$ ) was above 30, the t test is asymptotically equivalent to a z test [Sokal and Rohlf, 1981]. Thus, the resulting probabilities are independent of the degree of freedom. To compare the variabilities between the cell body frequency distributions (Fig. 6), we used variance analysis according to the F test [Sokal and Rohlf, 1981]. All data were entered and analyzed using functions implemented in Excel (Microsoft).

TABLE 3. Summary of the Experimental Series

Parameter	Series			
	1	2	3	4
Frequency [GHz]	no <sup>a</sup>	41.682–41.710	41.682–41.710	41.698
Power [ $\mu\text{W}/\text{cm}^2$ ]	no <sup>a</sup>	0.5	50	50
Temperature [°C]	27/30/33	30	28	30
$n^b$	1/7/2	15	19 <sup>c</sup>	6

<sup>a</sup>No indicates that no electromagnetic field was applied.

<sup>b</sup> $n$  is the number of independent determinations within a series to cover the whole frequency range by 2 MHz steps in series 2 and 3, at a given temperature in series 1, or the number of independent repetitions in series 4.

<sup>c</sup>Full range of frequencies ( $n = 15$ ) and 4 repetitions at 41.682 GHz, 41.684 GHz, 41.686 GHz, and 41.688 GHz with inversion of exposure versus control chamber (see Material and Methods).

## RESULTS

Control experiments (Table 3, series 1) showed that the cells were growing at a submaximal rate under the conditions chosen. Thus, deviations to slower and faster division rates could be observed. The sensitivity of the experimental protocol was demonstrated to be sufficient for detection of the reported differences between cells exposed and not exposed to EHF-EMF (see Introduction). Table 3 summarizes the experimental series performed to detect effects of EHF-EMF on cell division rate. Series 1 contains control experiments without field exposure. In series 2 and 3, the full frequency range from 41.682 to 41.710 GHz was covered at 2 MHz steps with two different power densities. The lower temperature in series 3 (28 °C instead of 30 °C) was chosen to leave more room to detect faster cell division. Both temperatures are physiological for *S. cerevisiae* [Sherman, 1991]. Series 4 is a sixfold repetition at 41.698 GHz.

### Control Experiments

The control experiments allowed assessment of a number of the conditions that were defined in the experimental design. First, undisturbed cell division was achieved. As an example, Figure 6A shows the primary data for S-phase cells from series 1, experiment 5, at 30 °C. Starting from single cells, with a small bud in this case, microcolonies with up to 23 cell bodies, corresponding to about four generations, developed after the incubation in the two chambers. The number of cell bodies was counted at  $t_1$  and was plotted in Figure 6A as the relative frequency of microcolonies with a given number of cell bodies. One cell or one bud is one cell body (see legend to Fig. 1). Thus, the buds of the last division were counted, although they had not yet separated from the mother cell. This method increased the sensitivity of the experiment and avoided a subjective bias of cell counting at  $t_1$ . The distributions for cells in chamber 1 and 2 were

superimposed for comparison. From all the experiments performed at 30 °C, we calculated a generation time of 87 min for G1-phase cells ( $n = 684$ ) and 80 min for S-phase cells ( $n = 762$ ). These values are very close and, if anything, are lower than the standard generation time (90 min) of *S. cerevisiae* in YPD at 30 °C [Sherman, 1991]. Therefore, the handling of the cells during spreading on agar and photography ( $t_0$  pictures) did not result in a measurable delay of cell division. The lower value for S-phase cells vs. G1-cells was expected, because S-phase cells have progressed further in the cell division cycle (see Fig. 1). The slightly faster generation times recorded here are best explained by our method of counting cell bodies at  $t_1$  after incubation (one cell or one bud is one cell body).

A second condition was cell growth at a submaximal rate, to allow observation of faster and slower division rates. Control experiments performed at 27 °C, 30 °C, and 33 °C demonstrated that this condition was met. For both G1-phase and S-phase cells, the mean length of an average cell cycle was higher at 27 °C (G1, 96 min; S, 88 min) than at 30 °C (G1, 87 min; S, 80 min), which was in turn higher than at 33 °C (G1, 80 min; S, 77 min). This temperature effect was expected according to yeast biology [Sherman, 1991]. Use of the z test on pairwise comparisons gave the following values: 33 °C vs. 30 °C,  $P = 6.77 \times 10^{-26}$  for G1 cells and  $P = 8.98 \times 10^{-12}$  for S-phase cells; 30 °C vs. 27 °C,  $P = 6.27 \times 10^{-14}$  for G1 cells and  $P = 1.83 \times 10^{-10}$  for S-phase cells. All these  $P$  values indicate extremely significant differences. Therefore, the EHF-EMF exposure experiments were performed at either 30 °C (series 2 and 4) or 28 °C (series 3), as indicated in Table 3.

The sensitivity of our experimental protocol was assessed as follows. The temperature control experiment comparing division rates at 30 °C and 33 °C resulted in a difference of 11% in average cell body number at  $t_1$ . This difference is detected as highly sig-

nificant in the statistical analysis ( $z$  test;  $P = 8.98 \times 10^{-12}$ ). Furthermore, analysis of the experiments in series 1 (Table 3) demonstrated that the variability between unexposed cells of a culture incubated in the two different chambers of the setup is already detected

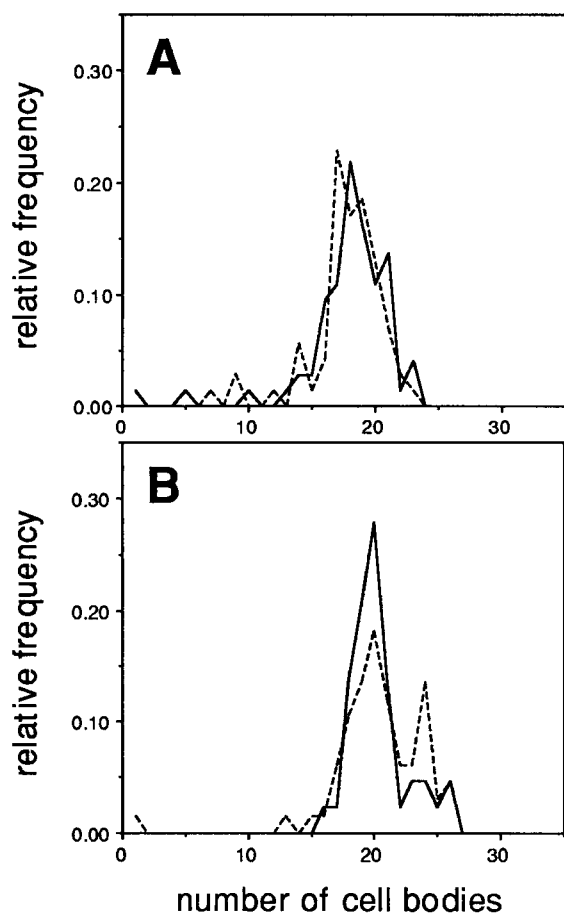


Fig. 6. Relative frequencies of the classes with a given number of cell bodies. Starting from single cells, the number of cell bodies was counted after incubation at  $t_1$ . The frequency of the distribution into classes with a given number of cell bodies was plotted as relative frequency over number of cell bodies for a particular class. **A:** Control experiment 5 without exposure in either of both chambers (series 1 of Table 3; no field). Solid line indicates distribution for cells in chamber 1 ( $n = 72$ ; mean = 18.208); dashed line indicates the distribution for the cells in chamber 2 ( $n = 70$ ; mean = 17.757). The results for S-phase cells are shown. The statistical analysis of this data point is shown in Figure 7 ( $z$  test,  $P = 0.3464$ ). **B:** Experiment at 41.708 GHz of series 2 (power density  $0.5 \mu\text{W}/\text{cm}^2$ ). Solid line indicates distribution for cells exposed to EHF-EMF ( $n = 43$ ; mean = 20.256); dashed line indicates the distribution for the concurrent unexposed control ( $n = 65$ ; mean = 20.631). The results for S-phase cells are shown. The statistical analysis of this data point is shown in Figure 9B ( $z$  test,  $P = 0.222$ ). Dead cells were included in the figure but were later excluded for statistical evaluation of the data (see discussion in Materials and Methods).

by our statistical methods. An example of the primary data for S-phase cells is shown in Figure 6A for experiment 5 of series 1. Figure 7 shows the results of the statistical analysis as  $z$  values for G1-phase and S-phase cells of all seven experiments of series 1. A number of  $z$  values are below the 5% level ( $P < 0.05$ ), a border usually chosen in biology to signal a significant difference. However, all  $z$  values were above the 1% level ( $P > 0.01$ ), with the single exception of S-phase cells in one experiment (series 1, experiment 7:  $z = -3.12$ ,  $P = 0.0023$ ; see Fig. 7). Thus, the biological variability in the experimental system is such that, with the  $z$  test, only  $P$  values of 1% or lower can be considered to indicate possible significant differences. With this 1% level, the experimental protocol can detect a significant effect on cell division rate even when the differences in the final cell body number were rather small. The 1% level of significance translates to a difference in average cell cycle length of less than 5 min (i.e., 5.6% of the standard generation time of 90 min). This high sensitivity results from incubating the cells for approximately four generations before the cell body number was determined. The control experiments of series 1 also established that there was no systematic difference between the two chambers of the exposure apparatus (see Fig. 7).

#### EHF-EMF Exposure of Exponentially Growing Cells

To detect a possible effect of EHF-EMF on exponentially growing *S. cerevisiae* cells, we have performed experiments in the frequency range from 41.682 GHz to 41.710 GHz using increments of 2 MHz. Figure 6B shows an example of the primary data for S-phase cells exposed at 41.708 GHz and the concurrent control (see Fig. 1 for the definition of S-phase cells). Starting from single cells with small buds, microcolonies developed after incubation in the exposure and the control chambers. The number of cell bodies was counted at  $t_1$  and was plotted in Figure 6B as the relative frequencies of microcolonies with a given number of cell bodies.

From the primary data, as shown in Figure 6B, a mean cell body number was calculated. The means for exposed and unexposed S-phase cells of series 2 are plotted in Figure 8, which shows a narrow distribution of the mean cell body numbers in all exposure experiments and controls, with the exception of the values for 41.684 GHz. In this experiment, the cells gave rise to a clearly higher cell body number than in the other experiments despite the standardized method of cell culturing. This effect was not caused by EHF-EMF, because the exposed and nonexposed cells gave similarly high values that were not significantly different

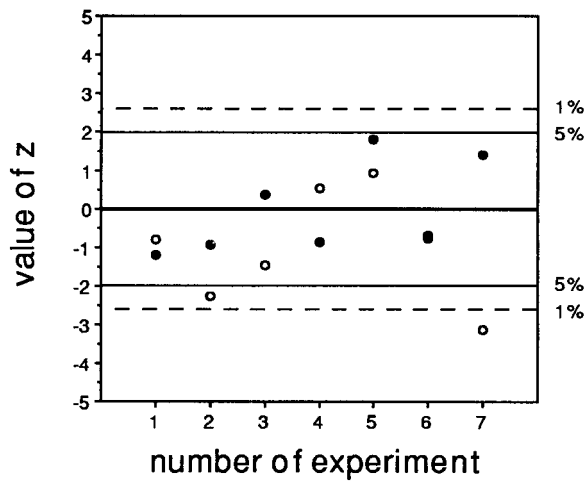


Fig. 7. Statistical analysis of mean cell body numbers of experimental series 1 at 30 °C (Table 3; no field). Plotted is the  $z$  value for the seven experiments for S-phase cells (open symbols) and for G1-phase cells (solid symbols). A thick line is drawn to indicate 0, a thin line is drawn to indicate the border from which  $z$  values can be considered statistically significant at the  $P = 0.05$  level, and a dashed line indicates the border for the  $P = 0.01$  level. A negative value for  $z$  reflects a lower mean for the cells in chamber 1, and a positive value for  $z$  reflects a higher mean for the cells in chamber 1.

from each other by statistical analysis ( $z$  test,  $P = 0.108$ ), as shown in Figure 9B. This example clearly shows the value of the concurrent control.

To compare the means of the cell body numbers between the concurrent exposures and controls in every single experiment, we employed statistical analysis as described in Materials and Methods. Figure 9 shows the results of experimental series 2 and 3, which both cover the entire EHF-EMF frequency range from 41.682 to 41.710 GHz. The two series differ in power density and incubation temperature (see Table 3). The value of  $z$ , the statistically relevant parameter for comparison of mean cell body numbers, was plotted in dependence of the EHF-EMF frequency. Only two values are below the 1% significance level that was set on the basis of the control experiments. These are the values for G1-phase cells at 41.682 GHz and for S-phase cells at 41.710 GHz, both in series 2 experiments. The former showed a difference of 15.7% in mean cell body number between exposed and unexposed cells ( $z$  test,  $P = 0.0068$ ). The latter value showed a difference of 10.3% in mean cell body number ( $z$  test,  $P = 0.0050$ ).

The experiments of series 4 (Table 3) were performed as multiple determinations at 41.698 GHz. This frequency was chosen because Grundler [1992] de-

scribed a particularly strong effect at this frequency. The experiments failed to reveal a consistent difference of mean cell body numbers at  $t_1$  between concurrently exposed and unexposed cells (Fig. 10). Pooling of the data from all 6 experiments also revealed no statistically significant difference (data not shown).

The above-mentioned data negate a possible effect of EHF-EMF on the average length of the cell division cycle in *S. cerevisiae*. However, it could be argued that some cells speed up their division cycle, whereas other cells within the same population would slow down their division cycle. The comparison of mean cell body numbers at  $t_1$  would not detect such a biological response to the EHF-EMF exposure because of the lack of a net effect at  $t_1$ . In this scenario, EHF-EMF exposure would result in broader distributions of cell body numbers at  $t_1$ . Therefore, we statistically compared the variances of final cell body number distributions of concurrent EHF-EMF exposures and controls.

Figure 11 shows the statistical analysis of the control experiment (Table 3, series 1); it displays the biological variability as the statistically relevant parameter  $F$  without application of EHF-EMF. The statistical test was extremely sensitive, with several values under the 5% limit ( $P = 0.05$ ). One value (experiment 5 S-phase cells of Fig. 11;  $F = 1.883$ ,  $P = 0.009$ ) was even below the 1% border. The values for G1-phase cells in this experiment exhibited no significant difference

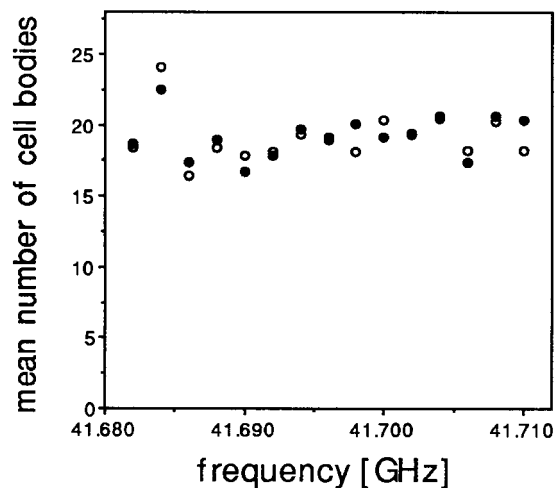


Fig. 8. Comparison of mean cell body numbers for exposed and unexposed control cells for the entire EHF-EMF frequency range. The results for S-phase cells for series 2 are displayed (see Table 3; power density  $0.5 \mu\text{W}/\text{cm}^2$ ). Open boxes indicate exposed cells and solid boxes indicate unexposed control cells. The pair at 41,684 GHz clearly shows the importance of the concurrent control; this culture obviously resulted in higher cell numbers for undetermined reasons.

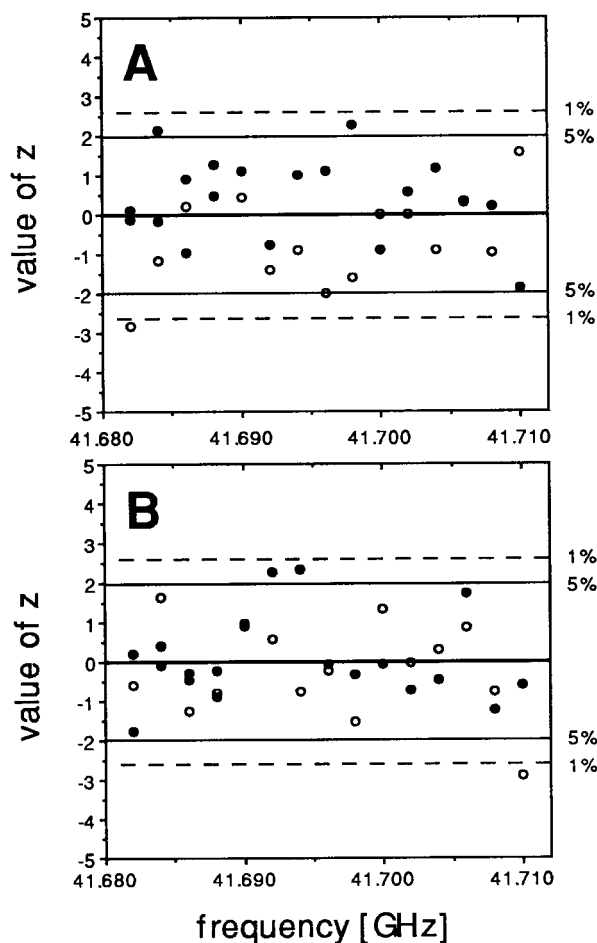


Fig. 9. Statistical analysis of the mean cell body numbers of experimental series 2 and 3 (Table 3; power densities  $0.5 \mu\text{W}/\text{cm}^2$  and  $50 \mu\text{W}/\text{cm}^2$ , respectively) for G1 cells (A) and for S-phase cells (B). Plotted is the  $z$  value for all experiments of series 2 (open symbols) and 3 (solid symbols) for the entire EHF-EMF frequency range. A thick line is drawn to indicate 0, a thin line is drawn to indicate the border from which  $z$  values can be considered statistically significant at the  $P = 0.05$  level, and a dashed line indicates the border for the  $P = 0.01$  level as indicated in the figure. A negative value for  $z$  reflects a lower mean for exposed cells, and a positive value for  $z$  reflects a higher mean for exposed cells.

( $P = 0.155$ ). Unlike the case in the  $z$  test (see Figs. 7, 9, 10), in the  $F$  test the  $P$  values for a given  $F$  are dependent on the degrees of freedom. Therefore, we cannot include  $P$  values in Figures 11–13 because there is no correlation with the absolute value of  $F$ . In conclusion, a detectable effect of EHF-EMF exposure resulting in increased variability of cell body numbers counted at  $t_1$  must produce  $F$  values yielding statistical significance consistently below the 1% level.

Figure 12 shows the statistical data for all experi-

ments in series 2 and 3 (Table 3) plotted as values of  $F$ , the statistical parameter for variance evaluation of cell body numbers, dependent on the tested EHF-EMF frequency. A number of  $F$  values were high enough to result in probabilities below  $P = 0.01$  and are indicated by an arrow in Figure 12. In the control experiment (Fig. 11), the 1% level was identified as the estimate of the biological variation. Between four and six  $F$  values were obtained for all the EHF-EMF frequencies tested, half of them for G1-phase cells, the other half for S-phase cells. Only at 41.698 GHz, two of four  $F$  values yielded a  $P$  value below 0.01 (series 2 S-phase cells, series 3 G1-phase cells). At all other EHF-EMF frequencies, only one  $F$  value or none from among four or six values resulted in  $P$  values below 0.01. At 41.698 GHz, these were for G1-phase cells in series 3 (Fig. 8A) and for S-phase cells in series 2 (Fig. 12B). The other two  $F$  values at this frequency yielded insignificant  $P$  values of 0.247 (G1-phase cells in series 2) and 0.219 (S-phase cells in series 3). The experiments in series 4 (Table 3) constitute six additional repetitions at this frequency of 41.698 GHz. The statistical data are represented in Figure 13. Only one of twelve values (S-phase cells experiment 6) indicated a difference in vari-

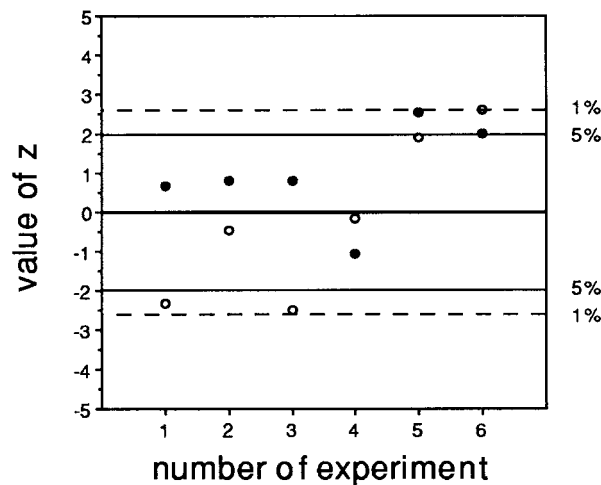


Fig. 10. Statistical analysis of the mean cell body numbers of experimental series 4 (Table 3; power density  $50 \mu\text{W}/\text{cm}^2$ ). Plotted is the  $z$  value for the six experiments of series 4 at 41.698 GHz for S-phase cells (open symbols) and for G1-phase cells (solid symbols). A thick line is drawn to indicate 0, a thin line is drawn to indicate the border from which  $z$  values can be considered statistically significant at the  $P = 0.05$  level, and a dashed line indicates the border for the  $P = 0.01$  level as indicated in the figure. A negative value for  $z$  reflects a lower mean for exposed cells, and a positive value for  $z$  reflects a higher mean for exposed cells. For experiments 1, 3, and 4 the exposure was in chamber 1, and for experiments 2, 5, and 6 the exposure was in chamber 2.

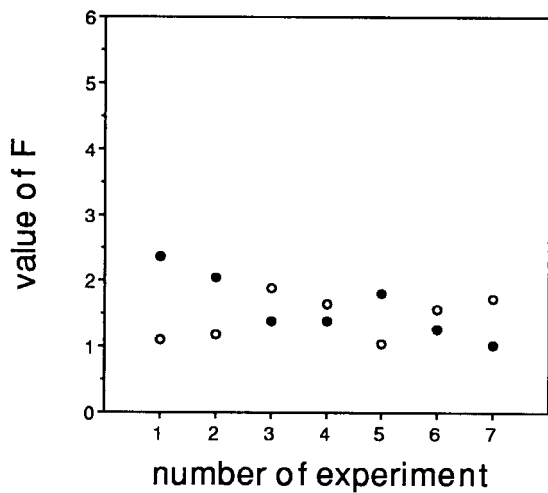


Fig. 11. Statistical analysis of the variability of cell body numbers in experimental series 1 (Table 3; no field). Plotted is the F value for the seven experiments of series 1 at 30 °C for S-phase cells (open symbols) and for G1-phase cells (solid symbols). Note that the absolute value of F is not correlated to the probability ( $P$ ). For further discussion, see text.

ability of cell body numbers at  $t_1$  beyond the natural variation documented by the control experiment (Fig. 11).

## DISCUSSION

This project was designed as an independent evaluation of a possible nonthermal effect of EHF-EMF on *S. cerevisiae* cell division rates previously observed by Grundler and colleagues (see References). Grundler and colleagues have described a biological effect of EHF-EMF at low power levels in the 42 GHz range on exponentially growing *S. cerevisiae* cells. Our experimental protocol maintained the reported experimental parameters as closely as was technically feasible. Our control experiments provided compelling evidence that the sensitivity of the setup is sufficient for detection of the reported changes in cell division rates: enhancement up to 108% and decrease down to 62% [Grunder, 1992; Grunder et al., 1992]. We calibrated the statistical tests by the control experiments and demonstrated that, owing to the biological variability, EHF-EMF effects could be detected only when differences between exposed and control cell populations were consistently significant at the 1% level. Control experiments with growth temperature variation have demonstrated that our experimental protocol was able to detect EHF-EMF effects that alter cell division rates by as little as 5%. It turned out to be essential that only data from concurrent field exposure and control experiments were compared. From all experimental series

with EHF-EMF exposure (Table 3, Figs. 9, 10, 12, 13), we conclude that there was no consistently significant biological effect of EHF-EMF on the division of *S. cerevisiae* cells. This has been demonstrated for two parameters: The first is directly related to average cell cycle length (division rate); the second assessed the variability of individual cell cycle lengths within the dividing cell population.

Scanning the frequency range from 41.682 GHz to 41.710 GHz at two different power levels yielded only two values, indicating an EHF-EMF effect on cell cycle length significant at the 1% level (Fig. 9). For G1 cells at 41.682 GHz of the second series, the differ-

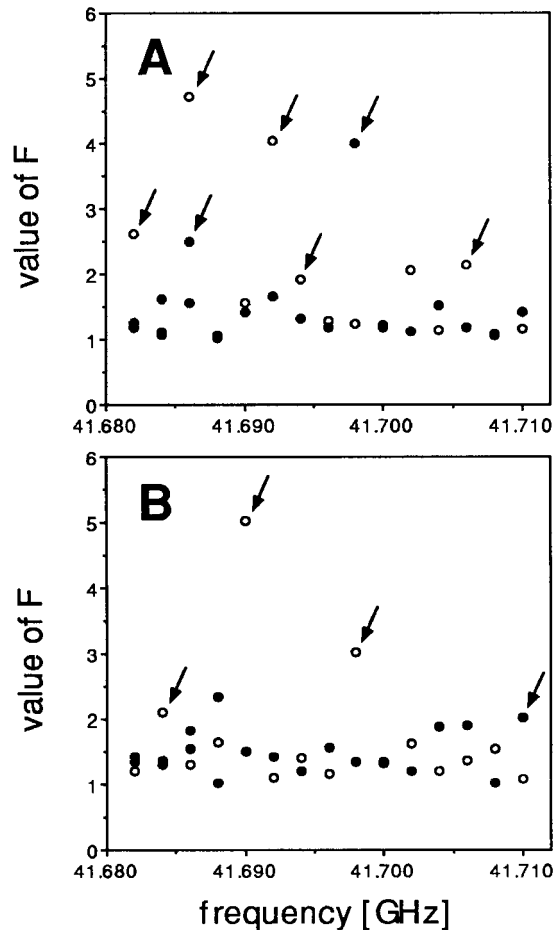


Fig. 12. Statistical analysis of the variability of cell body numbers in experimental series 2 and 3 (Table 3; power densities  $0.5 \mu\text{W}/\text{cm}^2$  and  $50 \mu\text{W}/\text{cm}^2$ , respectively) for G1 cells (A) and for S-phase cells (B). Plotted is the F value for all experiments of series 2 (open symbols) and 3 (solid symbols) for the entire EHF-EMF frequency range. Note that the absolute value of F is not correlated to the probability ( $P$ ). All F values resulting in a probability less than  $P = 0.009$  are marked by an arrow. This value was set by the control experiment shown in Figure 11 as the biological variation of the system. For further discussion, see text.

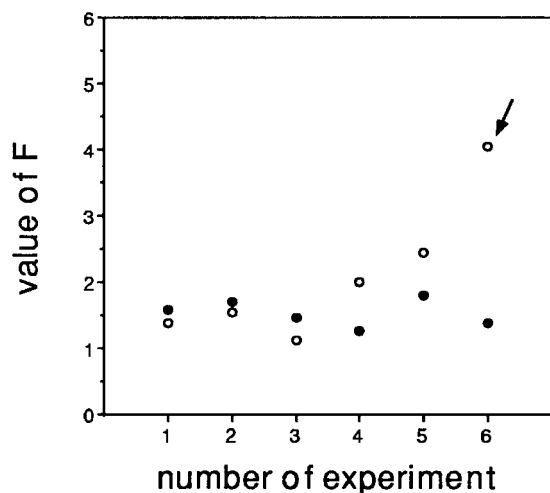


Fig. 13. Statistical analysis of the variability of cell body numbers in experimental series 4, six identical repetitions at 41.698 GHz (Table 3; power density  $50 \mu\text{W}/\text{cm}^2$ ). Plotted are the F values for S-phase cells (open symbols) and G1-phase cells (solid symbols). Note that the absolute value of F is not correlated to the probability ( $P$ ). One F value resulting in a probability less than  $P = 0.009$  is marked by an arrow. This value was set by the control experiment shown in Figure 11 as the biological variation of the system. For further discussion, see text.

ence in mean cell body numbers between exposure and control translates into a difference of 6.1 min per average cell cycle. For S-phase cells of the second series at 41.710 GHz, this translates into a difference of 2.4 min per average cell cycle. In the third series, no significant differences were detected at these frequencies (see Fig. 9). We ascribe the two significant differences to random variation. Likewise, one of seven control experiments (series 1, Fig. 7) exhibited a difference significant at the 1% level. In addition, the significant variations were seen only for one type of cell (G1 or S). A biologically significant effect should be exhibited by both types of cells, because they were actively growing during exposure. The distinction between G1- and S-phase cells led to a homogenization of the data and provided an additional internal control beside the concurrent control with unexposed cells.

The statistical analysis of average cell cycle length has revealed no biological effect of EHF-EMF, but one could construct an argument that the field affects individual cells in a given population differently. Specifically, this would mean that EHF-EMF causes some cells to speed up their cell cycle, whereas other cells would slow down their cell cycle. We have tested this hypothesis by statistical evaluation of the variance of cell body numbers at  $t_1$ . Again, the control experiments calibrated the sensitivity of the statistical analysis close to the 1% level. The observed significant dif-

ferences in variance must be ascribed to the natural variation of the biological system, because a consistent effect was not observed at any of the frequencies. A possible effect of EHF-EMF on the variability of cell cycle lengths at 41.698 GHz in the experiments of series 2 and 3 could not be reproduced by the experiments of series 4 at this same frequency. Therefore, we can exclude a consistently significant effect of EHF-EMF exposure on the variability of individual cell cycle lengths.

The conclusions presented here are obviously different from those of previous studies [Grundler et al., 1988; Grundler, 1989, 1992; Grundler and Keilmann, 1989] that we intended to confirm. A number of possible reasons for this difference are discussed below. (See also the discussion in the paper of Furia et al. [1986], describing a study that failed to reproduce independently a first series of experiments by Grundler and colleagues [Grundler et al., 1977; Grundler and Keilmann, 1978]. These studies had suggested a biological effect of EHF-EMF on *S. cerevisiae* cells grown in liquid culture.)

One explanation would be to ascribe the differences to the different *S. cerevisiae* strains used in our laboratory and in the previous studies. Such differences are commonly seen in *S. cerevisiae* for nonessential cellular functions and indicate the absence of selective pressure.

In addition, differences in other parameters might have affected the outcome of the present study. Possible differences in the whole experimental setup and some EHF-EMF parameters are discussed in Materials and Methods. Nevertheless, the salient parameters used by Grundler et al. have been kept constant: EHF-EMF frequency, power density, orientation of the chambers to the Earth's magnetic field, etc. Two relevant fundamental differences inherent in the present experimental protocol must be emphasized: 1) the question of control experiments in relation to rigorous statistical analysis and 2) the criteria for the choice of the cells to be analyzed.

The inherent value of the concurrent control is documented by the data shown in Figure 8 (see data for 41.684 GHz). In the protocol used by Grundler, historical controls were employed; i.e., data from different experimental cultures were compared. With the use of historical controls, it remains unclear how the natural variation in the biological system has been controlled. As all previous studies claimed, EHF-EMF effects relate to unexposed controls; the variation within these controls is of utmost importance for critical assessment with rigorous statistical methods. Important also are the criteria for the choice of cells for analysis. In the experimental protocol presented here, a clear set

of rules was defined, and all cells satisfying these criteria were analyzed. Otherwise, the experimenter might use unconscious subjective criteria for choosing cells and thus bias the study with unreproducible methods. We consider both of these points concerning the experimental protocol to be important and valuable improvements over the protocols used previously. They provide control over variations in the biological material and ensure unbiased data acquisition.

Beside these differences in the fundamental experimental protocol, a point relating to yeast biology is important. The fundamental claim in the previous studies has been a frequency-dependent positive or negative effect of EHF-EMF on the division rate of *S. cerevisiae*. This claim is particularly interesting in light of the advanced stage of yeast cell cycle research [reviewed in Forsburg and Nurse, 1991]; only very few mechanisms can be envisaged to speed up the cell cycle. Such mechanisms would involve cell cycle regulators such as the ones encoded by the *Schizosaccharomyces pombe cdc2* and *wee1* genes, for which certain mutations lead to premature cell division [Forsburg and Nurse, 1991].

In the previous studies, the cells had been synchronized by density centrifugation and stored at 4 °C for unspecified and varying times before experiments. Besides the problems of synchronizing cells, storage in the cold for a long time (days) in relation to the *S. cerevisiae* cell cycle (~90 min) will inevitably disturb the cellular physiology. In addition, it may lead to an exit of the cells from the mitotic cell cycle to a resting phase here termed G0 [Forsburg and Nurse, 1991] (Fig. 1). Thus, we suggest that it is possible that the transition from G0 to active cell cycle was analyzed in the previous studies. Because the length of time for which the cells were in G0 was not specified, this time difference leading to longer or shorter lag phases before resumption of exponential growth might well account for the observed differences in cell cycle length. The lack of concurrent controls makes this explanation even more likely, without invoking an effect of the electromagnetic field. However, the possibility remains that the transition from G0 to active cell cycle is sensitive to EHF-EMF fields. Future experiments with G0 cells prepared with a reproducible experimental protocol should shed light on this issue.

## CONCLUSIONS

A well-defined exposure system for experiments in the mm-wave range for microorganisms has been developed. Uniform, well-defined RF exposure characteristics for both control and exposed samples can be maintained over several hours in a temperature-stabi-

lized block. Electrical leakage between control and exposure samples has been determined to be better than 100 dB from several kHz up to 100 GHz. EHF-EMF in the 42 GHz range had no significant effect on the division of exponentially growing *S. cerevisiae* cells at power levels of 0.5 and 50  $\mu\text{W}/\text{cm}^2$ . The largest difference for the average length of the cell division cycle found in a single experiment was in the same range as the variation in control experiments. In addition, it can be excluded that EHF-EMF exposure increased the variability of individual cell cycle lengths within a population of dividing cells.

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