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Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates

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Abstract

The growth indicators 2,3,5-triphenyltetrazolium chloride (TTC), 2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride (INT), 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and resazurin were tested for their ability to indicate bacterial growth/growth inhibition. Two reading devices were evaluated and compared, a microplate spectrophotometer and a digital flatbed scanner. The bacteria used in the study were cultivated in 96-wells microplates and readings were made after 24 h. The scanned pictures were analysed with a software developed in-house to generate numerical values. It was found that resazurin was difficult to use since it shifts between three colours. MTT and TTC had a high correlation between the spectrophotometer data and the data from the scanned images. The reproducibility was similar for both reading devices. In no case was there a need to resuspend the pellets before reading. Both the XTT and INT showed lower correlations. It is concluded that bacterial growth/growth inhibition can be easily and reproducibly measured from microplate cultivations with a flatbed scanner or with a microplate spectrophotometer.

Keywords: Bacterial growth; Resazurin; Scanner; Tetrazolium salts

1. Introduction

Assessment of growth is often important when working with microorganisms, e.g. when investigating the antimicrobial effects of a compound. Microbial growth/inhibition of growth can be measured in a number of ways: plate counts (viable counts), direct microscopic counts, dry weight, turbidity measurement, absorbance, bioluminescence, etc. (Brock et al., 1994). When several strains are to be measured in parallel, it may be practicable to maintain, cultivate, and quantify them within 96-well microplates. Microbial growth in microplates is often measured as an increase in either absorbance (Gellert, 2000; Schrader et al., 1997) or in bioluminescence (Gellert and Stom-

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mel, 1999; Fuller et al., 2000). The added advantages of simplicity and high-throughput gained by using microplates are lost with the other quantification methods as the samples must be treated one by one. Furthermore, the use of microplates allows large amounts of data to be generated quickly and in one assay procedure.

Both measurement of bioluminescence and of absorbance have their drawbacks. Bioluminescence measurements are sensitive but demand relatively expensive equipment and can require extensive laboratory work prior to each assay. Absorbance measurements are easier to perform but are less sensitive and only valid within a limited concentration interval. A growth indicator, such as a tetrazolium salt or resazurin, may be added to facilitate the measurements (Bitton and Dutka, 1986; Botsford et al., 1997). The indicator is particularly useful when cells have a tendency to adhere together or where other additives affect the colour of the growth medium (Eloff, 1998). Traditionally, the quantification is made by absorbance measurement in a spectrophotometer.

Tetrazolium salts and resazurin have been used as growth indicators since the 1940s. They detect oxidative enzyme systems (Liu, 1981; Glenner, 1961) by acting as electron acceptors. The tetrazolium salts are dissolved and colourless in their native form but form nonwater, soluble, coloured salts as they are reduced. Resazurin is a coloured compound that changes colour (from blue to pink to colourless) but does not precipitate upon reduction.

The relation between the amount of precipitated salt/colour change and bacterial concentration is well established (Tengerdy et al., 1967; Mattila-Sandholm et al., 1991). The most common device for measuring the colour change is a spectrophotometer. It is an expensive device, especially when a microplate reader is needed. An attractive alternative to a plate spectrophotometer could be a flatbed scanner. A flatbed scanner offers advantages such as low price, ease of connection to a computer, and adaptability for other things than laboratory work. In the present study, the quantification of microbial growth inhibition through the use of a flatbed scanner has been evaluated. Comparisons were made between different tetrazolium salts and resazurin.

When inhibition of growth is quantified, certain criteria for the characteristics of the selected indicator have to be fulfilled. These may differ from the criteria when absolute growth is measured. Depending on its kinetics, the same indicator may be suitable for different conditions. If an instant assessment of the amount of bacteria in a solution is required, a fast-reacting indicator is demanded. Alternatively, if the effects of a chemical on the bacteria are to be studied, it might be more appropriate to use a slower-reacting indicator in order to let the bacteria have time to interact with the chemical. In addition, this opens up the possibility for kinetic studies during the course of growth. The choice of indicator also depends on which bacteria are used since not all indicators are able to be processed by some bacteria and therefore, do not respond to growth of these bacteria.

In the present study, we have evaluated the use of five different indicators, namely TTC, INT, XTT, MTT, and resazurin, for measuring relative growth/growth inhibition of microorganisms in microplates. Furthermore, the suitability of scanners and spectrophotometers as data-capture devices for detection of this inhibition was assessed.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study were either environmental strains of marine origin (here denoted

Table 1
Microbial strains used in the different plates

Column in microplate	Set I	Set II
1	*Vibrio fluvalis*	JG 11
2	*Aeromonas Hydrophila*	JG 50
3	*Escherichia coli* (MZ 480)	JG 52
4	*Enterococcus fecalis*	JG 53
5	*Enterococcus sulfureus*	JG 59
6	*Comamonas denitrificans* 110	JG 61
7	*Alcaligenes fecalis*	JG 62
8	*Vibrio alginolyticus*	JG 66
9	Oban F[a]	JG 71
10	*Saccharomyces cerevisiae*	JG 119
11	JG 63/119	JG 128
12	Blank	Blank

[a] Gram-positive coccus from Scotland.

JG) or known strains from culture collections. One yeast strain, *Saccharomyces cerevisiae*, was isolated from baking yeast (Jästbolaget, Sollentuna, Sweden). In summary, two sets of microorganisms were used during the study, denoted as Set I and Set II (Table 1).

2.2. Growth indicators

The following dyes were investigated: 2,3,5-triphenyltetrazolium chloride (TTC, tetrazolium red, Sigma T-8877, St. Louis, USA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma M-2128), 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt (XTT, Sigma X-4251), 2-[4-iodophenyl]-3-[4-dinitrophenyl]-5-phenyltetrazolium chloride (INT, Sigma I-8377), and resazurin (Eastman 2106, NY, USA).

2.3. Assay procedure

Round-bottomed 96-well microplates (Greiner, Frickenhausen, Germany) were used. To each well, 100 μl of nutrient broth (Difco Laboratories) supplemented with indicator was added. Unless otherwise

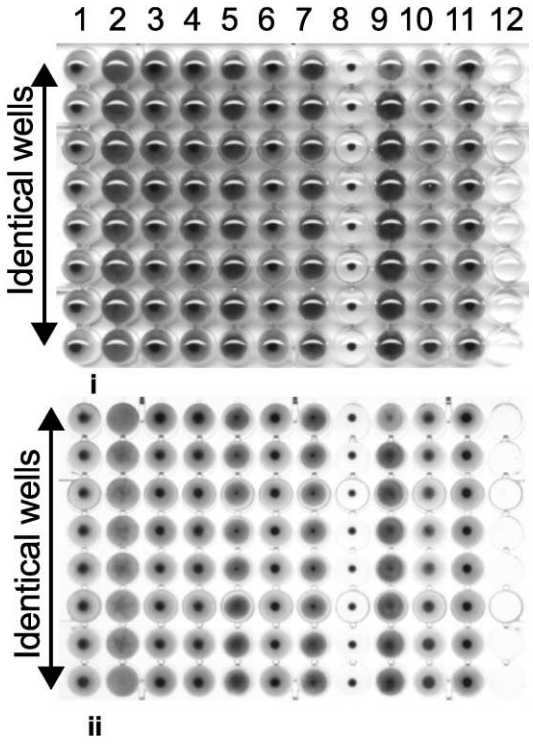


Fig. 1. Microplate with 11 different strains grown with tetrazolium red (TTC) as indicator. One bacterial strain was added to each of columns 1–11, bacteria as in Set II, Table 1. Different pellet shapes and densities were obtained. The bacteria were incubated at 28 °C overnight. (i) Microplate read with a scanner for reflectives. (ii) Microplate read with a scanner for transparencies. The pellets were measured with scanner (reflective and for transparencies)+in-house software and with a spectrophotometer at 540 nm. Coefficients of variation (CV) for the eight pellets from each of the 11 strains are given in the table. Similar mean values are obtained for all three devices. This is the base-line deviation that has to be encountered for when estimating the stability of the method.

Column/strain no.	1	2	3	4	5	6	7	8	9	10	11	Mean
Scanner, reflectives	0.03*	0.12	0.07	0.05	0.06	0.06	0.05	0.04	0.17	0.05	0.05	0.068
Scanner, transparencies	0.06	0.05	0.08	0.07	0.08	0.07	0.07	0.07	0.13	0.10	0.06	0.076
Spectrophotometr	0.03	0.06	0.08	0.08	0.08	0.06	0.04	0.07	0.17	0.07	0.05	0.072

* Coefficient of variation.

stated, the indicator concentration was 0.01% (w/v). A sterile toothpick was dipped into a single bacterial colony, cultivated on Nutrient agar (Difco Laboratories) at 28 °C overnight, and the colony was transferred into a microplate well. Alternatively, bacteria were freeze-dried (manuscript in preparation) in the microplates and stored at −20 °C for less than 3 months before the addition of the broth. Either methods of culture preparation gave the same results. The chemical compounds to be tested for their toxic effects were added at indicated concentrations, either as part of the nutrient broth or immediately after the addition of bacteria.

The microplates were incubated at 28 °C overnight. Although marine strains normally have a growth optimum below room temperature, this temperature was selected in order to avoid the use of a cooling incubator and 28 °C is a suitably low temperature that can be maintained in most laboratory incubators.

2.4. Reading of results

After incubation, the plates were read with a spectrophotometer (iEMS, Labsystems, Finland) at 492 or 540 nm. For TTC, 490 nm is the optimum according to the literature, but according to our results, 540 nm gave a higher resolution (data not shown). Alternatively, the plates were scanned with a reflective flatbed scanner (Agfa 600, Agfa-Gevaert, Mortsel, Belgium) or a transparency scanner (Arcus II, Agfa-Gevaert) connected to a Windows-based PC, according to the manufacturer's instructions. The microplates were covered with a white lid in order to enhance the contrast when using the reflective flatbed scanner. The scanned pictures were analysed using software developed in-house (available through PhPlate Microplate Techniques AB, Stockholm, Sweden). The software measured the intensity of the colour of the pellet as well as the diameter of it, resulting in a numerical value representing the amount of pellet which in turn represented the amount of bacterial growth in the well.

In one experiment using MTT as indicator, the pellets were dissolved in a solution of PBS (phosphate buffered saline, pH 7.5), 10% SDS (sodium dodecyl sulfate), and 10 mM HCl according to the manual from Roche (Cat No. 1465 007, F. Hoffmann-La Roche, Basel, Switzerland) before reading the result. The reading was made at 540 nm.

2.5. Computations

Coefficient of variation (CV) and Pearson's momentum correlation coefficient were used to compare the quantification methods.

When quantifying inhibitory effects during this study, the inhibition of microbial growth has been measured not as absolute values but as relative values. That is, the growth in the wells containing an additional chemical was compared with the control, "blank" wells containing only broth, bacteria, and growth indicator.

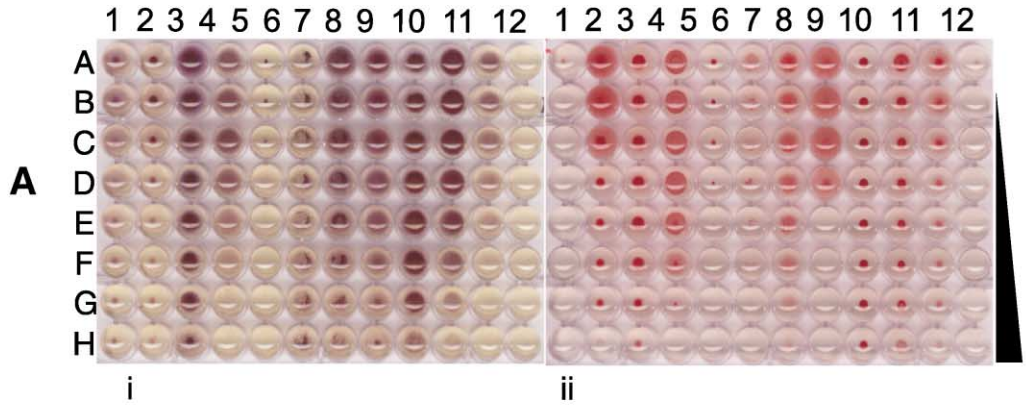
The lowest observed effect level (LOEL) is a commonly used term in risk assessment. It is the lowest level at which any inhibitory effect can be observed. Another term often found is the IC_X-value, the concentration which gives X% inhibition of growth. The LOEL-values shown in this study were obtained by visual inspection of the images, noting the concentration at the appropriate well (i.e. the first well where inhibition of growth was seen) while the IC_X-values were calculated by interpolation between raw data.

3. Results and discussion

3.1. Pellet shape

A characteristic of the measurement of bacterial growth using TTC or MTT was that different bacterial strains produced different pellet shapes (Fig. 1) upon reduction of the growth indicator. Some strains gave a small, distinct pellet confined to the bottom of the well (e.g. column 8, strain JG66), whilst other bacterial strains gave a dispersed pellet with the insoluble reduced form of TTC distributed across a larger area of the well (e.g. column 2, strain JG50). Upon micro-

Fig. 2. Comparison between readings done with a digital flatbed scanner and readings done with a spectrophotometer. Microplates with chemical gradients were read with both devices. The graphs show the correlation between the two readings (arbitrary units, au). The correlation is somewhat higher with thiazolyl blue (MTT) than with tetrazolium red (TTC). Plate (i) 11 strains (Set I, Table 1), MTT, ethanol gradient (Fig. 5i). Plate (ii) 11 strains (Set I, Table 1), TTC, acrylamide gradient.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A
B
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F
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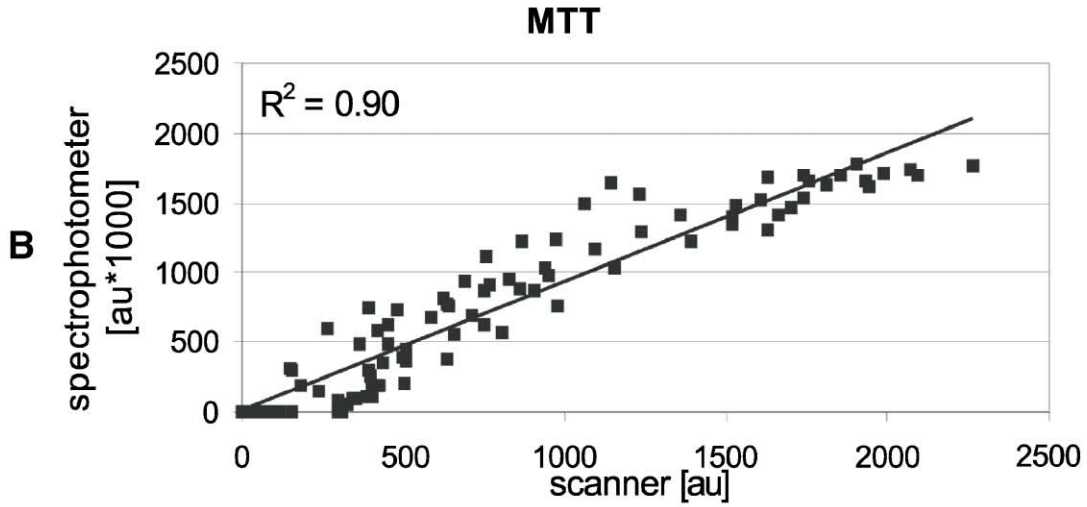
H
i
ii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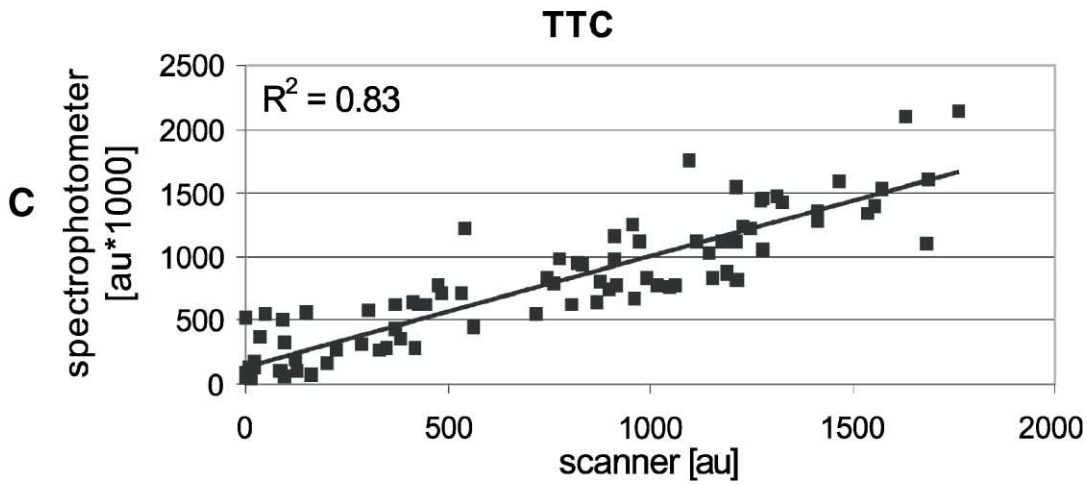
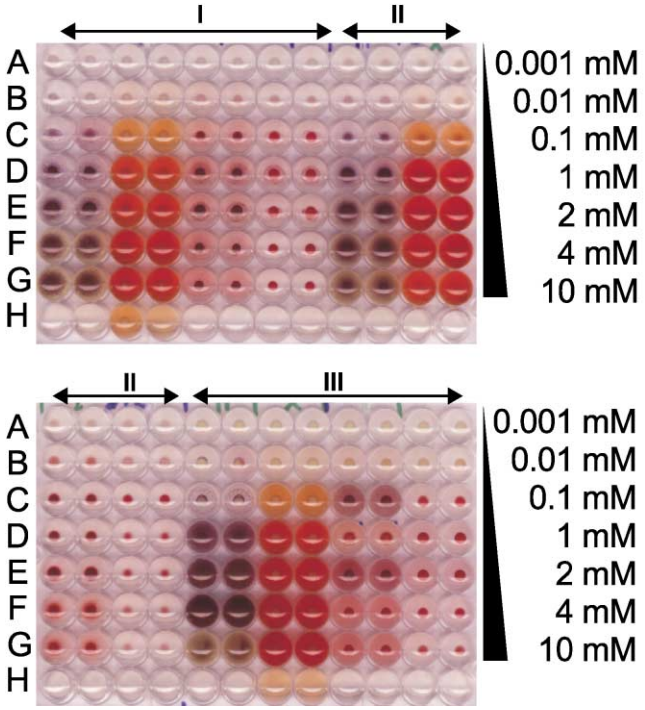
MTT
R2 = 0.90
spectrophotometer [au*1000]
scanner [au]
0
500
1000
1500
2000
2500


C
TTC
R2 = 0.83
spectrophotometer [au*1000]
scanner [au]
0
500
1000
1500
2000
2500

scopic examination, it was clear that the strains giving a distinct pellet appeared to be immotile whilst strains giving a dispersed pellet displayed a motile phenotype. When looking at the variation (CV) between the wells containing the same isolate, there was no relation between the shape of the pellet and its reproducibility with any of the tested reading devices (Fig. 1): with, e.g. the spectrophotometer strain JG50 (column 2, dispersed pellet) and JG66 (column 8, small distinct pellet), gave a CV of 0.06 and 0.07, respectively.

3.2. Correlation between scanner and spectrophotometer

The raw data obtained upon reading with the spectrophotometer and with the scanner showed a good correlation, 0.83–0.90 (Fig. 2). The correlation was higher when MTT was used as an indicator than when TTC was used. A closer analysis of the data showed that data captured using the scanner yielded comparatively higher values for the small pellets, as shown in column 9 (Fig. 2ii), than data captured using the spectrophotometer. This deviation leads to a lower coefficient of correlation if the precipitated growth indicator has a higher tendency to form sharp and distinct pellets with some bacteria. This was the case with TTC compared to MTT, and therefore, the correlation appeared lower. As to which kind of data best represents the bacterial growth is, however, difficult to ascertain. On the other hand, wells with dense bacterial growth showed a tendency to yield lower values with the spectrophotometer when MTT was used (Fig. 2B). Measurements of a plate containing mostly large dispersed pellets (Fig. 1) yielded a correlation of 0.89 between reflective scanner and spectrophotometer. The reproducibility is similar between the two reading devices (Fig. 1).



No	Strain
I	*E.coli*
II	*Vibrio alginolyticus*
III	Oban F (a Gram+ cocci)

Fig. 3. Comparison of different concentrations of growth indicators. Microplates containing concentration gradients of MTT, XTT, INT, and TTC (0.001–10 mM from row A to row G, approximately 0.00005–0.5% (w/v) depending on the indicator) and three different bacterial strains (I *Escherichia coli*, II *Vibrio alginolyticus*, III Oban F-a gram+cocci). Row H contains the same concentration of growth indicator as row C (0.1 mM) but no bacteria.

3.3. Transparency scanner

The pellets appeared more distinct and clearer using the transparency scanner than using the reflective scanner and in addition, they did not suffer from the "smile-like" reflections that were inevitable as results when scanning round-bottomed microplates with a reflective scanner (Fig. 1). Despite this, the

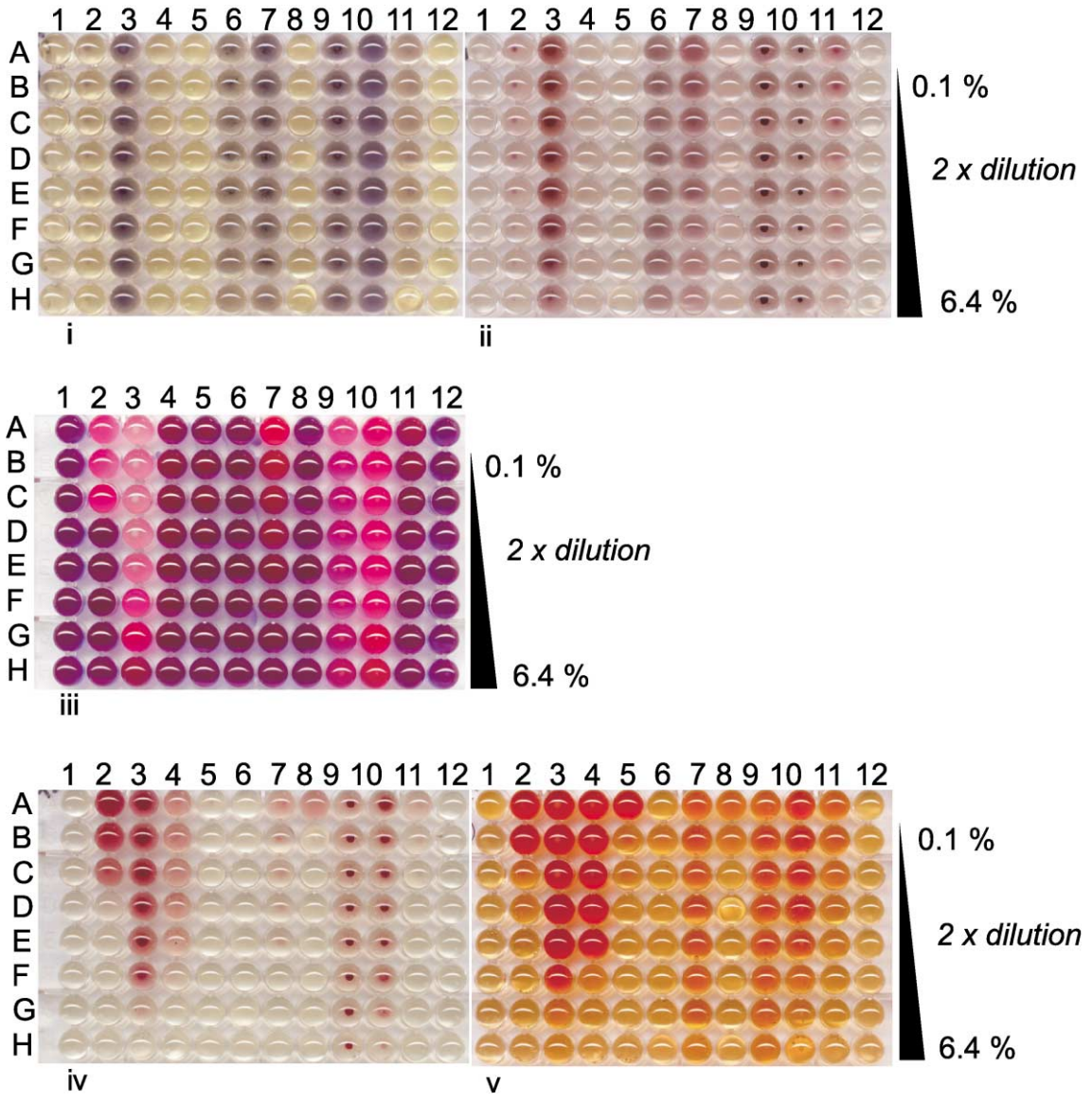


Fig. 4. Comparison between different growth indicators used for detection of growth inhibition due to toxicants. Microplates contained 11 different strains, one in each of columns 1–11, and a gradient of acrylamide (0.1–6.4% from rows B to H, 2×dilution steps). The first (top) row in each plate constitutes a "blank", i.e. it contains no toxicant, and thus all microbial strains grow there. No indication of growth in a column indicates that the actual strain cannot reduce the specific indicator. Bacteria as in Set I, Table 1. (i) MTT, (ii) INT, (iii) resazurin, (iv) TTC, (v) XTT.

pellets gave the same variation (CV), which in average is about 7%, when measured with a spectrophotometer as when measured with a scanner (reflective or transparency) (Fig. 1). Similar CV-values were obtained for plates using MTT as growth indicator (data not shown). Lower CV-values were obtained if the plates were kept in a moist chamber during incubation.

3.4. Advantages with a scanner

The use of a scanner instead of a spectrophotometer offers a number of advantages such as low cost (a factor of 20–40×), ease of connection to a computer, and availability. A transparency scanner is slightly more expensive than a reflective scanner. One drawback with the scanners is that they require more computer storage memory since images instead of numbers are to be saved. This is just a temporary problem though; as soon as the images have been analysed, they may be deleted.

3.5. Comparison to more laborious methods

Mattila-Sandholm et al. (1991) found that corresponding CV-values for plate counting and turbidimetry measurement were about 0.06 (0.053–0.075, somewhat higher for the plate-count technique). Similar CV-values were found in the present study (Fig. 1). This may constitute a minor source of error compared to other error sources in an assay. It should also be noted that scanners were not designed for three-dimensional microplates but flat sheets of paper. Variation in the result may be due to positioning on the scanner,

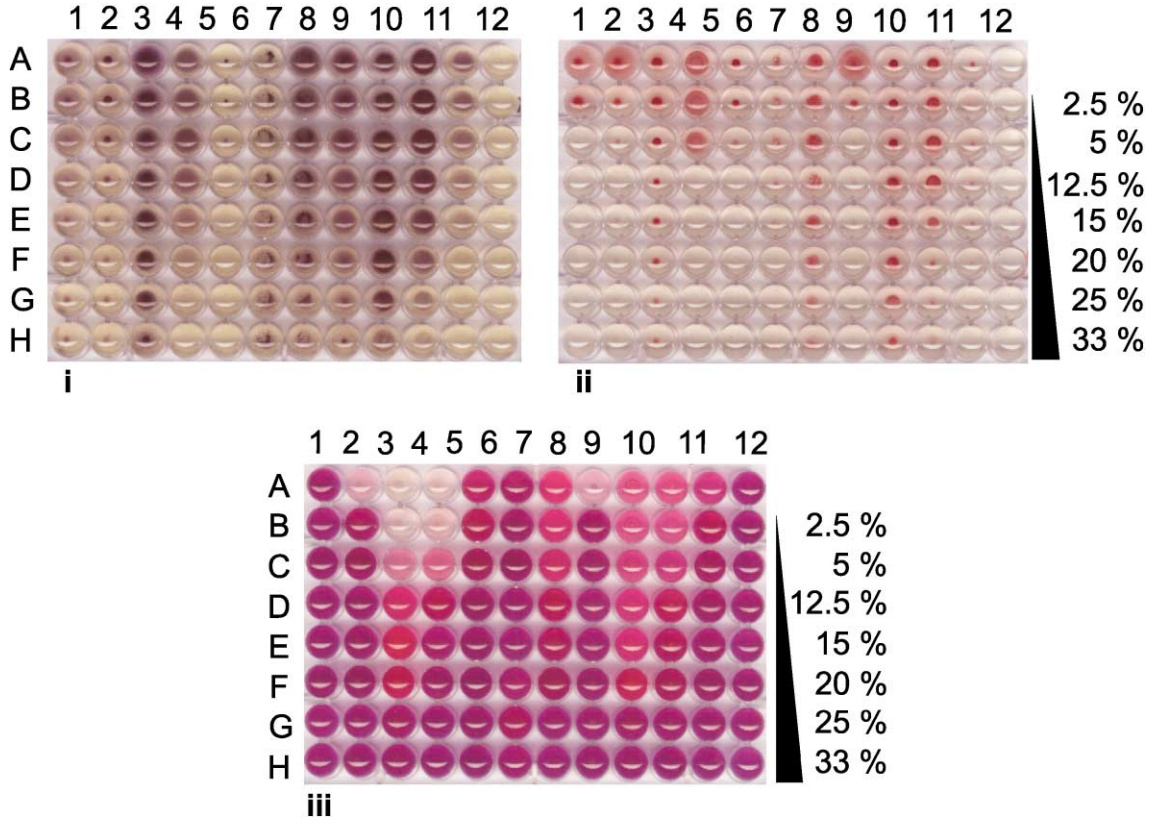


Fig. 5. Comparison between different growth indicators used for detection of growth inhibition due to toxicants. Microplates contained 11 different strains, one in each of columns 1–11, and a gradient of ethanol (2.5–33% from rows B to H). The first (top) row in each plate constitutes a "blank", i.e. it contains no toxicant, and thus all microbial strains grow there. No indication of growth in a column indicates that the actual strain cannot reduce the specific indicator. Bacteria as in Set I, Table 1. (i) MTT, (ii) TTC, (iii) resazurin.

variations in the intensity of the light bulb, external laboratory lightning, etc. In addition, results from the scanner are also dependent on the algorithm in the software that analyses the images.

3.6. Type of plates

Compared to flat-bottomed microplates, round-bottomed microplates enhance the pellet formation of precipitating growth indicators and they have thus been used throughout these assays. When scanning round-bottomed microplates with a reflective scanner, light reflections give rise to small "smile-like" reflections in each well. This disturbance does not affect the calculation of the growth as the in-house software compensates for them by measuring the diameter above the reflection instead of the whole area of the pellet. Another advantage with round-bottomed wells is that smaller volumes are required than in flat-bottomed wells. As in ELISA, border effects have been noted (i.e. a weaker growth in the outermost wells). Again, these effects can be minimised if the plates were incubated in a moist chamber.

3.7. Indicator concentration

An assay using different indicator concentrations (MTT, XTT, INT, TTC) (Fig. 3) showed no significant differences in pellet size/colour for concentrations ranging from 1–10 mM (~0.05–0.5% w/v) for any of the indicators. Below 0.1 mM (~0.005%), the reading of the indicator was unreliable as the pellet size diminished with decreasing indicator concentration. The result was similar for all three tested bacterial strains. A wide range of indicator concentrations has been used in other studies. Eloff (1998) found that the lowest effective concentration of TTC is 0.2% while Botsford et al. (1997) uses 0.0005% [w/v]. It is desirable to minimise the indicator concentrations since the indicator may interfere both with bacterial growth and with other chemicals added to the growth media. On the other hand, a certain excess of growth indicator in the solution is required so that the amount of indicator does not constitute a limiting factor for the maximal size of the pellet/colour of the solution. During the assays presented in this paper, the lowest possible concentrations according to our own results (Fig. 3) were used, i.e. 0.01% [w/v].

3.8. Growth inhibition

Inhibition of bacterial growth was tested by adding a concentration gradient of acrylamide or ethanol to a microplate containing different microbial strains (Figs. 4 and 5) A comparison between five different growth indicators with regards to their ability to detect inhibition of microbial growth is presented in Figs. 4 and 5 and Tables 2 and 3. Bacterial growth

Table 2
Comparison of sensitivity of inhibition

Column/strain no.	1	2	3	4	5	6	7	8	9	10	11
(A) LOEL-values, visual readings of the microplates in Fig. 4											
MTT	–	0.4	>6.4	–	–	1.6	1.6	–	>6.4	>6.4	6.4
INT	–	0.2	3.2	–	–	3.2	3.2	–	>6.4	>6.4	0.4
Resazurin	–	0.1	1.6	–	–	–	0.1	–	0.1	3.2	–
TTC	–	0.1	0.8	0.8	0.1	–	0.1	0.1	6.4	0.1	0.1
XTT	0.1	0.2	1.6	1.6	0.1	–	6.4	0.1	3.2	1.6	0.4
(B) IC_{10}-values, data from computerised analysis of the scanned microplates in Fig. 4 *using 10% pellet reduction in order to obtain an interpolated value*											
MTT	–	0.25	>6.4	–	–	0.9	2.5	–	1.6	>6.4	3.3
INT	–	<0.1	4.3	–	–	12.3	2.5	–	>6.4	>6.4	1.4
Resazurin	–	0.11	<0.1	–	–	–	–	–	<0.1	>6.4	–
TTC	–	<0.1	0.3	0.15	–	–	0.15	<0.1	>6.4	<0.1	–
XTT	–	0.12	1.2	1.0	<0.1	–	–	–	0.8	0.95	0.1

Comparison of sensitivity for inhibition, i.e. ability to detect inhibition of bacterial growth, between indicators. Inhibition values, given as concentrations (% w/v) for each strain and indicator in Fig. 4. The "–" indicates that the strain has not affected the indicator.

Table 3
Comparison of sensitivity of inhibition

Column/strain no.	1	2	3	4	5	6	7	8	9	10	11
(A) LOEL-values for ethanol, visual readings of the microplates in Fig. 5											
MTT	5	5	2.5	12.5	5	>33	12.5	15	25	15	5
TTC	2.5	2.5	5	5	2.5	2.5	20	2.5	33	15	2.5
Resazurin	–	2.5	5	2.5	5	–	5	2.5	20	12.5	2.5
(B) IC_{10}-values for ethanol, data from computerised analysis of the scanned microplates in Fig. 5 *and from reading of the same plates with a spectrophotometer, using 10% pellet reduction in order to obtain an interpolated value*											
MTT scanner	<2.5	3.1	3.8	6.5	2.9	>33	8.6	3.4	21.5	9.8	<2.5
MTT spectrophotometer	<2.5	3.7	<2.5	11.3	8.6	>33	20	<2.5	22	5.2	<2.5
TTC scanner	<2.5	<2.5	3.2	4.2	<2.5	<2.5	7	<2.5	24.4	<2.5	<2.5
TTC spectrophotometer	<2.5	<2.5	2.7	<2.5	<2.5	<2.5	<2.5	<2.5	20	3.2	<2.5

Comparison of sensitivity for inhibition, i.e. ability to detect inhibition of bacterial growth, between indicators. Inhibition values, given as concentrations (% w/v) for each strain and indicator in Fig. 4. The "–" indicates that the strain has not affected the indicator.

inhibition was easily detected visually with TTC and XTT. Furthermore, TTC detected a higher sensitivity of the bacteria to toxic effects of the tested chemicals compared to MTT (13 strains of 17), INT (6 strains of 6), and XTT (6 strains of 9), and appeared equal to resazurin in this regard. A "higher sensitivity" means that the growth inhibition of a specific strain is detected at a lower concentration of the tested chemicals. Some combinations of strain and indicator are not very successful and may lead to difficulties in the determination of growth. This explains some of the differences in result between the indicators. However, resazurin had the drawbacks of being reduced by fewer of the tested strains and could not be easily read by the spectrophotometer at a single wavelength since it displayed three different colours (blue–pink–colourless) during transformation. In addition, the variations (or nuances) in the purple colouration, although visible by eye, were not detectable using a scanner. TTC, however, gave clear-cut results both visually by spectrophotometer and by scanner.

The indicator of choice will principally be determined by the method of measurement. If a flatbed scanner and the type of software that has been used in this study are to be used, a tetrazolium salt giving a pellet (i.e. MTT, TTC, or INT) is recommended. The three salts have different properties such as reaction kinetics or ability to detect growth of a specific strain. For testing of the growth inhibition capacity of chemicals on an array of microbial strains, TTC was the best choice. However, XTT and resazurin could be more attractive choices with an adapted software that can read colour ranges, such as Bionumerics from Applied Maths (Gent, Belgium). The price is another important factor, TTC is by far the cheapest of the tested indicators followed by resazurin, while XTT constitutes an expensive alternative.

3.9. Dissolved pellets

Cell growth can be measured with MTT by dissolving the pellet and reading the absorbance in the vial with a spectrophotometer (Roche "cell proliferation kit"). Spectrophotometer readings of dissolved and nondissolved MTT pellets in the microplate were compared (data not shown). A better reproducibility was obtained with nondissolved pellets than with dissolved pellets for a majority of the tested bacteria. It is an advantage if this step can be avoided, since it would both reduce the amount of work in the assay and the risk of contamination when adding another component to the bacterial solutions.

4. Conclusion

A flatbed scanner offers a good alternative to a microplate spectrophotometer when measuring relative microbial growth/inhibition of growth with a tetrazolium salt such as TTC or MTT as indicator in microplates. This may allow the development of inexpensive toxicity assays that could be used much more widely than existing systems.

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