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Article *in* The Science of The Total Environment · September 2023

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Inoculum cell count influences separation efficiency and variance in Ames plate incorporation and Ames RAMOS test

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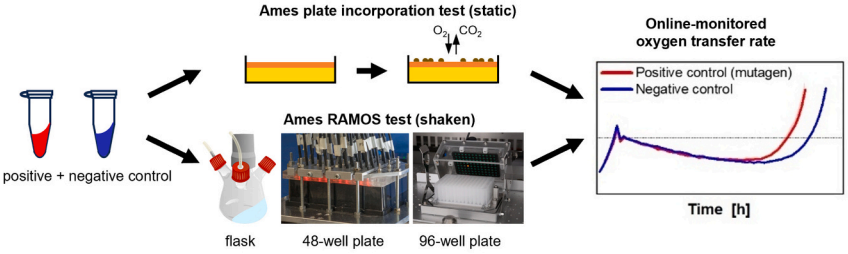
HIGHLIGHTS

- Area-specific oxygen transfer rate monitored during Ames plate incorporation test
- Oxygen transfer rates comparable to the Ames RAMOS test
- Ames RAMOS test progressed identically in flask, 48-well and 96-well MTP scale.
- Higher inoculum cell count led to a lower coefficient of variance in both tests.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Daqiang Yin

Keywords:
Ames test
Mutagenicity
Oxygen transfer rate
Salmonella typhimurium

ABSTRACT

The Ames test is one of the most applied tools in mutagenicity testing of chemicals ever since its introduction by Ames et al. in the 1970s. Its principle is based on histidine auxotrophic bacteria that regain prototrophy through reverse mutations. In the presence of a mutagen, more reverse mutations occur that become visible as increased bacterial growth on medium without histidine. Many miniaturized formats of the Ames test have emerged to enable the testing of environmental water samples, increase experimental throughput, and lower the required amounts of test substances. However, most of these formats still rely on endpoint determinations.

In contrast, the recently introduced Ames RAMOS test determines mutagenicity through online monitoring of the oxygen transfer rate. In this study, the oxygen transfer rate of *Salmonella typhimurium* TA100 during the Ames plate incorporation test was monitored and compared to the Ames RAMOS test to prove its validity further. Furthermore, the Ames RAMOS test in 96-well scale is newly introduced. For both the Ames plate incorporation and the Ames RAMOS test, the influence of the inoculum cell count on the negative control was highlighted: A lower inoculum cell count led to a higher coefficient of variation. However, a lower inoculum cell count also led to a higher separation efficiency in the Ames RAMOS test and, thus, to better detection of a mutagenic substance at lower concentrations.

Abbreviations: 2-AA, 2-aminoanthracene; CV, coefficient of variation; FAU, Formazine attenuation units; LLOD, lower limit of detection; μTOM, Micro(μ)-scale Transfer rate Online Measurement device; MTP, micro titer plate; NC, negative control; OTR, oxygen transfer rate; OTR′, area-specific oxygen transfer rate; PC, positive control; RAMOS, respiratory activity monitoring system.

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https://doi.org/10.1016/j.scitotenv.2023.167035
Received 6 July 2023; Received in revised form 29 August 2023; Accepted 11 September 2023
Available online 12 September 2023

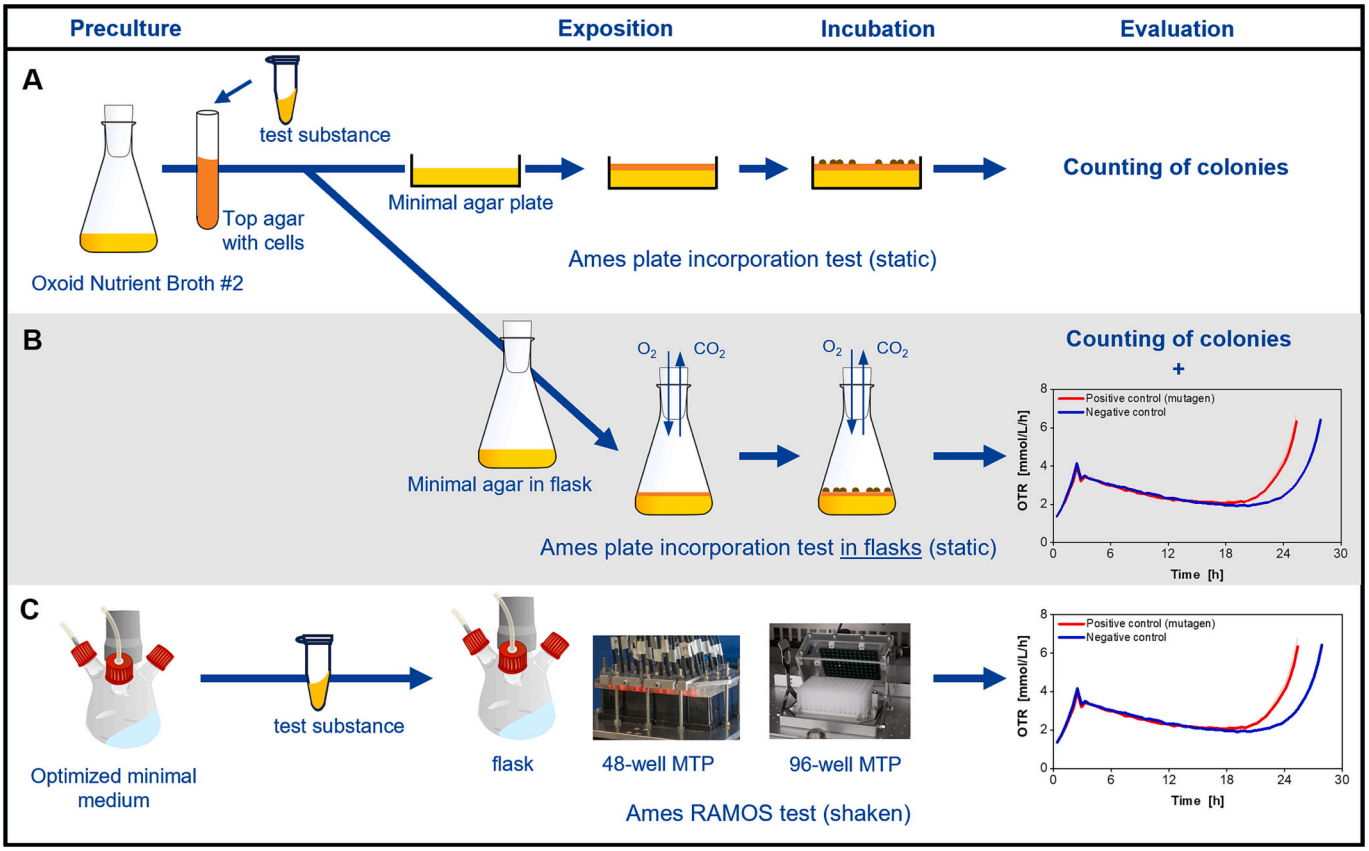


Fig. 1. Graphical illustration of the Ames plate incorporation test performed offline and online as well as the Ames RAMOS test. (**A**) Ames plate incorporation test: Final colony counts are noted for data evaluation. (**B**) Online measurement of the Ames plate incorporation test: The test is carried out in RAMOS flasks. The flasks are connected to a RAMOS device, where the oxygen transfer rate (OTR) is monitored over 48 h of static incubation. Evaluation takes place via colony counts in the flasks and via online OTR data. (**C**) Ames RAMOS test performed in three scales: Flask scale, 48-well scale, and 96-well scale. Evaluation takes place only via the online OTR data as described by Kauffmann et al. (2020b).

1. Introduction

The Ames test, or "bacterial reverse mutation assay" (Gatehouse, 2012), "*Salmonella* mutagenicity assay" (Claxton et al., 2010; Mortelmans and Zeiger, 2000) or "reversion assay" (Skopek et al., 1978) is used worldwide for the evaluation of potential mutagens in environment and industry (Mortelmans, 2019; Zeiger, 2019). Since its development by Ames et al. (1975) and Maron and Ames (1983), it has become a "stethoscope of genetic toxicology" (Claxton et al., 2010). For environmental water samples, the test is performed according to ISO 11350 (2012), with its relevance increasing after the revision of the EU water framework directive (Brack et al., 2018; Brack et al., 2019). In addition, the Ames test is required to assess chemicals as part of the first in vitro test battery in Japan, China, Korea (Ji et al., 2017) and the European Union (2006). For regulatory purposes, the Ames test is performed according to OECD 471 (1997) in either the "plate incorporation" or the "preincubation" version. In both cases, five strains are chosen from a strain library and grown on agar plates. The test strains are histidine-auxotrophic *Salmonella typhimurium* or *Escherichia coli* containing specific histidine operon mutations (McCann et al., 1975; Mortelmans and Zeiger, 2000). During the Ames test, cells can regain prototrophy through spontaneous or induced mutations and can, thus, grow on a minimal medium. The prototrophic cells become visible as colonies on an agar plate and are counted by the naked eye to evaluate a mutagenic effect (Ames et al., 1975; Maron and Ames, 1983).

Many laboratories that perform the Ames test use their own miniaturized test formats to prescreen substances in higher throughput and for a faster determination of suitable test concentrations (Escobar et al., 2013). Miniaturized test formats are also preferred in cases where very little test sample is available. In this context, tests in 6-, 24- and 25-well agar plate format (Brooks, 1995; Burke et al., 1996; Côté et al., 1995; Diehl et al., 2000; Escobar et al., 2013; Flamand et al., 2001; Proudlock and Evans, 2016) have been developed that each use a different selection of Ames test strains (see overview in Table S1). All these test formats roughly follow the original Ames test protocol. A second group of miniaturized Ames tests are performed in submerged form, mostly to enable automatization and to save laboratory space (e.g., Ames II and Ames microplate format (MPF) assay). These test variants consist of a preincubation phase in 24-well format and an incubation phase in 384-well format, where revertants are detected with the help of a pH indicator (Flückiger-Isler et al., 2004; Flückiger-Isler and Kamber, 2012; Green et al., 1976; Kamber et al., 2009; Spiliotopoulos and Koelbert, 2020). The main weakness of these miniaturized tests is the lower inoculum per plate/well, and thereby, they are more likely to miss weak responses as coefficients of variation (CV) are reported to range from 20 to 100 % (Egorova et al., 2020; Flamand et al., 2001; Pant et al., 2016; Reifferscheid et al., 2012).

For the testing of environmental water samples, a simplified protocol known as the Ames fluctuation test has been implemented in ISO 11350 (2012) and extensively validated (Reifferscheid et al., 2012). Amongst others, the Ames fluctuation test is applied to determine the mutagenicity of sewage and wastewater (Akhtar et al., 2016; Pérez et al., 2003), as well as drinking water (Lv et al., 2015; Roubicek et al., 2020), and freshwater samples (Shuliakevich et al., 2021). The test is only performed with two test strains (*S. typhimurium* TA98 and TA100) since this two-strain combination can detect over 90 % of the mutagens detected by a full Ames test performed with five strains (Williams et al., 2019). The lowest effect concentrations, at which tested chemicals can still be

detected with different Ames test formats (Flückiger-Isler and Kamber, 2012; Proudlock and Evans, 2016; Rainer et al., 2021; Reifferscheid et al., 2012) and negative/vehicle control ranges (Kato et al., 2018; Pant et al., 2016; Reifferscheid et al., 2012) have been extensively described in literature. Many recent publications have focused on strategies and criteria for evaluating Ames test results (Hayashi, 2022; Kirkland et al., 2007; Levy et al., 2019; Zeiger, 2023). However, the influence of the respective Ames test protocols on the separation efficiency and coefficient of variance (CV) has not been discussed so far.

An alternative type of Ames test, the Ames RAMOS test in shake flasks and 48-well microtiter plates (MTP), has recently been presented by Kauffmann et al. (2020b). The test protocol builds on the Ames fluctuation test and applies only the strains TA98 and TA100. Thus, it is suited for tests of both chemicals and environmental water samples. In contrast to previous test systems, the mutagenic effect is not determined from an endpoint evaluation, such as colony or well counting. Instead, the auxotrophic and prototrophic bacteria are quasi-continuously monitored via their oxygen transfer rate (OTR). The respiration activity monitoring system (RAMOS) used to monitor the test has been established for over 20 years (Anderlei et al., 2004; Anderlei and Büchs, 2001). Over this time, the RAMOS technology has been proven to monitor mammalian, plant cell, and microbial respiration activity across a wide range of cultivation conditions and media in a robust and reliable way (Ihling et al., 2022; Scheidle et al., 2007; Schulte et al., 2018; Wewetzer et al., 2015). Through online monitoring of the OTR during the Ames RAMOS test, the test duration has been reduced by almost 50 % to 24–30 h compared to conventional Ames test protocols that employ endpoint measurements. Furthermore, reproducibility has been improved by optimizing the preculture protocol and lowering the cultivation temperature (Kauffmann et al., 2020b). While the Ames test on agar plates has extensively been modeled (Krewski et al., 1993; Stead et al., 1981) and a general kinetic model for the Ames RAMOS test was introduced by Kauffmann et al. (2020a), a direct comparison between the two systems has not yet been presented. Specifically, the transferability of the Ames RAMOS test readouts to colony counts has yet to be defined.

This study compares the Ames RAMOS test and the Ames plate incorporation test directly by online monitoring the area-specific oxygen transfer (OTR′) of agar plates. In addition, further miniaturization of the Ames RAMOS test to a 96-well MTP is demonstrated, and the online OTR signals for all three scales (flask, 48-well, 96-well) are compared. Finally, the influence of the inoculum cell count on the negative control and its CV is investigated, and a trade-off between CV and separation efficiency is presented.

2. Material & methods

2.1. Microorganism

The Ames test strain *S. typhimurium* TA100 was purchased from Trinova Biochem. The same lot was used for all experiments performed in this study. Cryo cultures were only thawed once.

2.2. Media

2.2.1. Complex medium for the preculture of the Ames plate incorporation test

Oxoid nutrient broth #2 (purchased in liquid form from Trinova Biochem) was used for the preculture. Ampicillin was added to a 50 μg/mL concentration immediately before use.

2.2.2. Minimal agar for the Ames plate incorporation test

Minimal agar (Vogel-Bonner minimal medium E with D-glucose) and overlay agar were prepared according to Maron and Ames (1983). The overlay agar contained 8.7 mg/L L-histidine and 11.1 mg/L biotin for some initial cell divisions.

2.2.3. Minimal medium for the Ames RAMOS test preculture

The preculture for the Ames RAMOS test was conducted in an optimized minimal medium, which was adapted from ISO 11350 by Kauffmann et al. (2020b). It contained 4.3 g/L D-glucose, 2.2 g/L citric acid monohydrate, 10.8 g/L K_2HPO_4, 3.8 g/L $NaNH_4HPO_4{\cdot}4\ H_2O$, 2.8 g/L $MgSO_4{\cdot}7\ H_2O$, 2.6 mg/L D-biotin and a trace element solution originally described by Wilms et al. (2001). The trace element solution consisted of 0.11 mg/L $ZnSO_4{\cdot}7\ H_2O$, 0.10 mg/L $CuSO_4{\cdot}5\ H_2O$, 0.06 mg/L $MnSO_4{\cdot}H_2O$, 0.11 mg/L $CoSO_4{\cdot}7\ H_2O$, 8.36 mg/L $FeCl_3{\cdot}6\ H_2O$, 0.40 mg/L $CaCl_2{\cdot}2\ H_2O$ and 6.69 mg/L Na_2Ethylendiaminetetraacetic acid (EDTA)$\cdot 2\ H_2O$. The pH value of the medium was adjusted to a value of 7.0 with NaOH. For the preculture, 20 mg/L L-histidine and 50 μg/mL ampicillin were added.

2.2.4. Minimal medium for the Ames RAMOS test exposition

For the combined exposition and incubation phase of the Ames RAMOS test, the minimal medium described above was used with a 1.2-fold increased total concentration to sustain longer bacterial growth during the test. Only 5 mg/L L-histidine and no ampicillin were added.

2.3. Ames plate incorporation test

An illustration of the methodology is presented in Fig. 1A–B. The Ames plate incorporation test was performed in 9 cm Petri dishes and simultaneously in 250 mL flasks with respiration online monitoring (respiration activity online monitoring system, RAMOS) using a measurement strategy introduced recently by Finger et al. (2023a).

20 mL minimal agar was distributed into Petri dishes and 250 mL RAMOS flasks.

The preculture was conducted overnight. Two 250 mL flasks were each filled with 20 mL of complex medium, inoculated with 80 μL from a cryo culture, and shaken at 37 °C. Shaking conditions were 250 rpm and 50 mm shaking diameter. After stopping the preculture, the optical density was measured with a photometer (Genesys™ 20, Thermo Fisher Scientific), and the cell count was determined using a particle counter (Multisizer 4, Beckman Coulter).

Per Petri dish/flask, 2 mL of overlay agar tempered to 40 °C were mixed with 0.1 mL test substance, 0.5 mL S9 mix or phosphate buffer, and 0.075 mL cell suspension taken from the overnight culture, resulting in approx. 2×10^9 cells per plate. The mixture was then distributed on the agar in both Petri dishes and RAMOS flasks and allowed to solidify at room temperature. Subsequently, Petri dishes and flasks were incubated at 37 °C under static conditions for 48 h in the same incubation hood. RAMOS flasks were connected to an in-house built RAMOS device (Anderlei et al., 2004; Anderlei and Büchs, 2001), which provided one measurement point for the area-specific oxygen transfer rate (OTR′) every 45 min.

After 48 h of incubation, the agar plates in Petri dishes were scanned using an Epson Perfection V700 photo scanner (Epson). RAMOS flasks were photographed with a Google Pixel 5A (Google). To ensure the reproducibility of the following steps, a 2 mm stage micrometer (Ernst Leitz Wetzlar GmbH) was included in every picture as a size standard. Colonies were counted automatically from a representative area, using the ImageJ 1.53 k (Schneider et al., 2012) functions “Auto threshold” and “Analyze particles,” as described by Finger et al. (2023b) and validated with manual counts. A minimum area of 5 cm^2 was analyzed.

2.4. Ames RAMOS test

The Ames RAMOS test was performed in the optimized version described by Kauffmann et al. (2020b) for flask and 48-well scale and adapted to 96-well scale. An illustration of the methodology is given in Fig. 1C. For each preculture, 20 mL of optimized minimal medium (Kauffmann et al., 2020b) was inoculated with 200 μL of cryo culture and cultivated at 30 °C in a 250 mL RAMOS flask. The flask was shaken at 250 rpm with a diameter of 50 mm. The preculture was stopped once

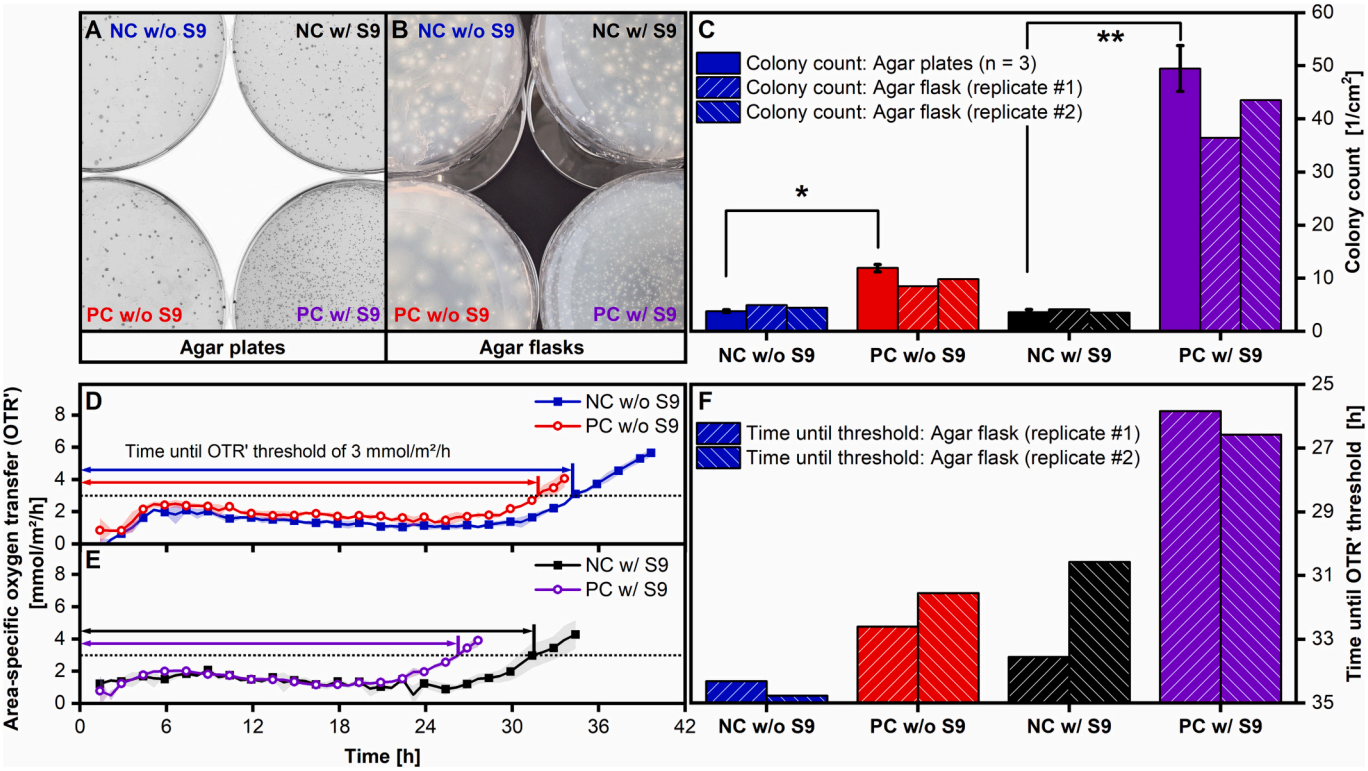


Fig. 2. Comparison of offline and online Ames plate incorporation test results (**A**) Ames plate incorporation test performed with *S. typhimurium* TA100 in Petri dishes according to Fig. 1A (cf. OECD 471, n = 3, full picture in Fig. S1). NC w/o S9 = negative control (DMSO), PC w/o S9 = positive control (sodium azide), NC w/ S9 = negative control with metabolic activation via S9 mix (DMSO), PC w/ S9 = positive control with metabolic activation via S9 mix (2-aminoanthracene). (**B**) Ames plate incorporation test performed in 250 mL flasks, as illustrated in Fig. 1B (n = 2, full picture in Fig. S2). (**C**) Colony counts after 48 h of incubation (Table S1). Statistical significance against negative control determined via *t*-test, * $p < 0.05$, ** $p < 0.01$. (**D**) and (**E**) Area-specific oxygen transfer rate (OTR′) over time for the Ames plate incorporation test without (**D**) and with (**E**) metabolic activation via S9 mix. Test performed in 250 mL flasks with *S. typhimurium* TA100, according to Fig. 1B. Mean of duplicates, every third data point indicated by a symbol, shadows indicate minimum and maximum. (**F**) Time until OTR′ = 3 mmol/m^2/h (horizontal dotted lines in (**D**) and (**E**)) is reached.

histidine limitation became visible through the drop of the OTR signal, as introduced by Kauffmann et al. (2020b). Optical density was determined using a photometer (Genesys™ 20, Thermo Fisher Scientific). Then, the preculture was diluted to 45 ± 5 Formazine attenuation units (FAU) using 1.2-fold concentrated optimized minimal medium. For some experiments, the preculture was diluted to 70, 90, or 105 ± 5 FAU instead. The positive control, negative control, or a test substance was added in a volumetric ratio of 1:50. The mix was distributed into the respective cultivation vessel and incubated at 30 °C. The lower temperature compared to 37 °C of the Ames plate incorporation test had previously been shown to improve the separation efficiency (Kauffmann et al., 2020b).

2.4.1. Flask scale

Each 250 mL RAMOS flask was filled with 20 mL of cell suspension. The OTR was monitored using an in-house built RAMOS device (Anderlei et al., 2004; Anderlei and Büchs, 2001). The shaking conditions were 250 rpm at a shaking diameter of 50 mm.

2.4.2. 48-Well scale

Each well of a 48-well microtiter plate (MTP) with round wells (Beckman Coulter, formerly m2p labs) was filled with 2.4 mL of cell suspension and enclosed with a sterile barrier (900371-T, HJ-Bioanalytik). The OTR was monitored using an in-house built μRAMOS device (Flitsch et al., 2016). The shaking conditions were 700 rpm at a shaking diameter of 3 mm.

2.4.3. 96-Well scale

Each well of a 96-well deep well plate (J.T.Baker® Plate Medio 2 mL, Avantor) was filled with 1 mL of cell suspension and enclosed with a sterile barrier (AeraSeal™, Excel Scientific). The OTR was monitored using a Micro(μ)-scale Transfer rate Online Measurement device (μTOM) device (Dinger et al., 2022). The shaking conditions were 1000 rpm at a shaking diameter of 3 mm.

2.5. Test substance and controls

All positive controls (PC) were dissolved in Dimethyl sulfoxide (DMSO) to a stock concentration of 1 g/L, aliquoted, and stored at −20 °C. A fresh aliquot was thawed for each experiment and diluted with DMSO to a working concentration 50-fold higher than the respective final test concentration. The working solutions were added at a volumetric ratio of 1:50 to the Ames RAMOS test or at 0.1 mL/plate to the overlay agar of the Ames plate incorporation test in both Petri dishes and flasks.

The positive control substances for the Ames plate incorporation test were chosen according to OECD 471 (1997): In both Petri dishes and flasks, sodium azide at a final test concentration of 0.5 μg/plate was used as the positive control without metabolic activation. 2-Aminoanthracene (2-AA) at a final test concentration of 2.5 μg/plate was used as a positive control for the Ames plate incorporation test conditions with metabolic activation (S9). S9 fraction (rat, beta-naphtoflavone and phenobarbital-induced) was obtained from Trinova Biochem and stored at −80 °C. The S9 mix was prepared according to Reifferscheid et al. (2012) and added to the overlay agar at a volume of 0.5 mL/plate.

In all Ames RAMOS tests, nitrofurantoin was used as the positive control at a final test concentration of 0.25 mg/L. The control complies with ISO 11350 (2012) to ensure comparability to the Ames fluctuation

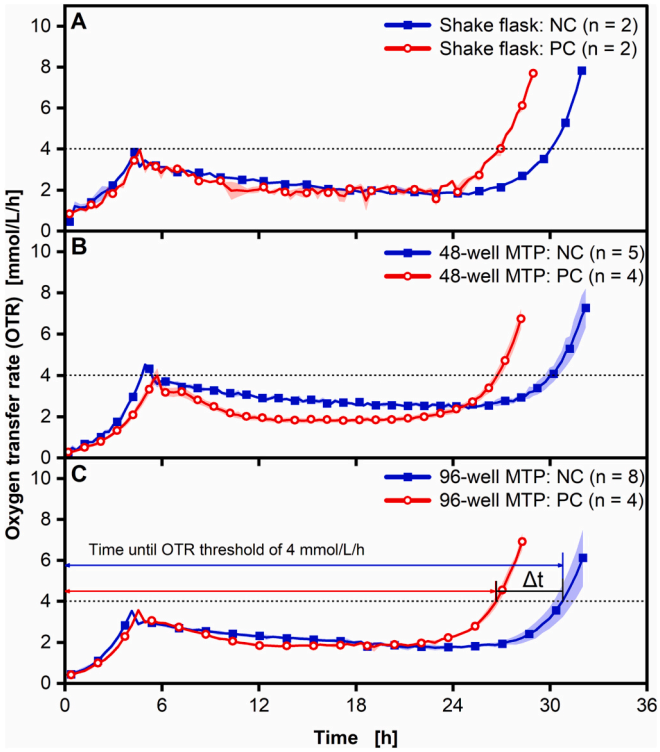


Fig. 3. Oxygen transfer rate (OTR) of *S. typhimurium* TA100 over time in three scales for the Ames RAMOS test (compare Fig. 1C). Initial optical density was set to 45 ± 5 Formazine attenuation units (FAU) in 1.2-fold concentrated optimized minimal medium containing 5 mg/L histidine (see Section 2.2.3). NC = negative control without metabolic activation (DMSO), PC = positive control without metabolic activation (0.25 mg/L nitrofurantoin). Symbols indicate every fifth data point; shadows indicate standard deviation. Horizontal dotted lines indicate the threshold value (OTR = 4 mmol/L/h) used to evaluate the data. Δt = separation efficiency between positive and negative control. (**A**) Shake flask: 20 mL in 250 mL flasks, 250 rpm shaking frequency, 50 mm shaking diameter. (**B**) 48-well MTP: 2.4 mL in 48-well RoundPlate, 700 rpm shaking frequency, 3 mm shaking diameter. (**C**) 96-well MTP: 1 mL in 96-well Deep Well Plate (round), 1000 rpm shaking frequency, 3 mm shaking diameter.

test.

In agreement with OECD 471 (1997) and ISO 11350 (2012), respectively, pure DMSO was added as the negative control (NC) with the same ratios/volumes as the positive controls.

2.6. Evaluation of online OTR and OTR′ data for the Ames plate incorporation and Ames RAMOS test

As previously shown and validated by Kauffmann et al. (2020a, 2020b), the OTR signal for an Ames RAMOS test without any mutagen is expected to progress identically to the NC in Fig. 1C. In contrast, the OTR signal for an Ames RAMOS test in the presence of a mutagen is expected to increase earlier than that of the NC (see the OTR of the PC in Fig. 1C). The mutagenicity of a sample can thus be determined through the time-shift of the OTR increase between a sample and the NC.

The online OTR and OTR′ data were evaluated using a MATLAB® script (Mathworks, Inc.). To quantify the time-shift of the OTR/OTR′ increase, the script read out at which timepoint the OTR/OTR′ first intersected with a threshold (visualized by a dotted line in Fig. 1C) after histidine depletion. For the Ames RAMOS test, the threshold was set to OTR = 4 mmol/L/h (Kauffmann et al., 2020b). The threshold for the Ames plate incorporation test was set to OTR′ = 3 mmol/m^2/h.

Depending on the measurement setup, measurement points for the OTR and OTR′ were only recorded every 20–45 min. To model the course of the OTR and OTR′ in between measurement intervals, an exponential fit was generated for a range of five measurement points. From this exponential fit, the time $t_{threshold}$, where the OTR or OTR′ equals the respective threshold, was calculated. In the last step, the separation efficiency Δt was calculated according to Eq. (1).

$$\Delta t = t_{threshold}(NC) - t_{threshold}(test\ substance) \quad (1)$$

3. Results and discussion

3.1. Investigation of oxygen transfer during the Ames plate incorporation test

The area-specific oxygen transfer (OTR′) of *S. typhimurium* TA100 during the Ames plate incorporation test was monitored in a RAMOS device similar to Finger et al. (2023a). *S. typhimurium* TA100 was chosen as a representative strain because it is considered one of the most sensitive *Salmonella* test strains (Margolin et al., 1981; Williams et al., 2019). As the goal of this study was mechanistic insight, four conditions were tested: PC positive control (PC) and NC negative control (NC) with (w/) and without (w/o) S9. OECD 471 (1997) recommends DMSO as the negative control and sodium azide (w/o S9) as well as 2-aminoanthracene (w/ S9) as positive controls. Thus, these substances were chosen as textbook examples. Since the RAMOS device only permitted eight parallel measurements, each condition was monitored in duplicates. To increase the number of replicates, every condition was additionally tested in triplicates in regular Petri dishes.

Fig. 2 shows pictures of the revertant colonies formed on agar in regular Petri dishes (A) and RAMOS flasks (B). To compensate for the difference in surface area due to the different diameters of the vessels, the average number of colonies per cm^2 was used for the evaluation (Fig. 2C; absolute colonies per plate are given in Table S1, Supplementary data). The colony counts for both Petri dishes and RAMOS flasks agree very well with each other. The difference in colony counts between PC and NC of the test performed in Petri dishes was significant (*t*-test, $p > 0.05$ for condition w/o S9, $p > 0.01$ for condition w/ S9), proving the validity of the Ames test. No statistical test was performed on the colony counts in glass flasks, as only duplicates were available. While colony counts for the test controls vary widely across literature, all results are within the reported range for *S. typhimurium* TA100 (Diehl et al., 2000; Pant et al., 2016).

For all monitored test conditions, the online OTR′ signal (Fig. 2D–E) initially increases to a value between 2 and 3 mmol/m^2/h. During this time, *S. typhimurium* TA100 grows on the supplied amount of histidine and, thus, uses up more and more oxygen. After histidine depletion, the histidine-auxotrophic cells cannot grow further but are still actively respiring. The cells are slowly lysing, resulting in a gradual decrease of the OTR′ for all conditions. During this time, prototrophic revertant colonies start to grow but are not yet visible in the sum signal of the OTR′. Only after 20 h, the revertant respiration activity is high enough to form an increase of the OTR′ (for the PC w/ S9). The OTR′ then reaches the threshold of 3 mmol/m^2/h (dotted line) after 26.5 h. However, the OTR′ of the respective NC w/ S9 reaches the same threshold at approx. 32 h. The points in time of the OTR′ reaching the threshold are indicated for each separate flask in Fig. 2F. They correspond qualitatively to the respective colony counts shown in Fig. 2C. A higher colony count indicates a higher number of revertants at histidine depletion, resulting in a head start for revertant growth, which becomes visible in the earlier OTR′ signal increase.

A similar oxygen transfer rate (OTR) trend has been shown and modeled for the Ames RAMOS test (Kauffmann et al., 2020a). However, in the case of the Ames RAMOS test in submerged, continuously shaken format, the OTR forms a sharp peak at histidine depletion (Fig. 3). Since on solid agar, cells and medium are not mixed during cultivations, nutrients are available in different amounts to different cells. Only the cells on the surface of a colony grow aerobically and consume oxygen. In contrast, the cells on the inside of a colony are covered by other cells and

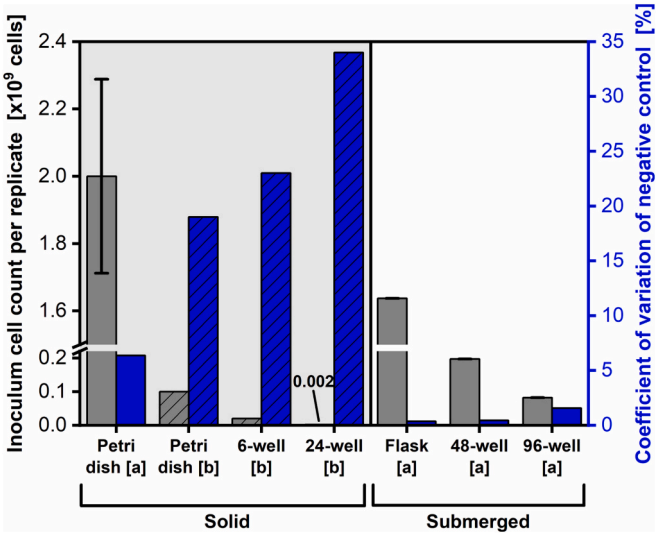


Fig. 4. Comparison of initial cell count (grey) and coefficient of variation (CV, blue) of the negative control. Left: Solid cultivation on agar plates. Right: Submerged cultivation in liquid medium. [a] data from this work; [b] data from Pant et al. (2016). The approximate cell count of the submerged scales was calculated from the inoculation cell density of 45 FAU multiplied by the respective filling volume: 20 mL in a flask, 2.4 mL per well in a 48-well MTP and 1 mL per well in a 96-well MTP. Error bars indicate the standard deviation for the data generated in this work ([a]). CV of negative control was calculated for the respective readouts: Colony count for solid agar cultivations; Cultivation time in hours until OTR = 4 mmol/L/h in submerged scales.

experience anaerobic conditions (Tschiersch et al., 2012). As anaerobically growing cells do not consume oxygen, their growth cannot be monitored through the oxygen transfer. Therefore, the OTR′ signal only shows the respiration of the aerobically growing cells on the surface of each colony. As the colonies on an agar plate may have different sizes and surface areas at a given time, the OTR′ signal consists of overlapping, heterogenous respiration activity (Finger et al., 2023a). In contrast, a permanently mixed culture broth would result in homogenous growth where all cells respire aerobically to the same extent and, thus, a more defined OTR peak can be expected. However, the general trajectories of the OTR during the Ames RAMOS test and the OTR′ during the Ames plate incorporation test agree well with each other. Not only does it mechanistically validate the Ames RAMOS test, but it also opens up the option to compare colony counts and OTR readouts.

3.2. Comparison of the Ames RAMOS test across flask, 48-well, and 96-well scale

So far, the Ames RAMOS test has been introduced in flask and 48-well scale (Kauffmann et al., 2020a, 2020b). In this study, the test format was transferred into 96-well scale, which doubles the experimental throughput. A comparison of the OTR progression for *S. typhimurium* TA100 of PC (red, empty symbol) and NC (blue, full symbol) for all three scales is shown in Fig. 3. To keep the Ames RAMOS test comparable to the Ames fluctuation test, nitrofurantoin was used as the PC following ISO 11350 (2012).

Overall, the OTR curves of both PC and NC agree well across scales. As previously shown in a mechanistic model by Kauffmann et al. (2020a), the OTR reveals valuable information about the growth status of the test strain cultivation. Since the medium is permanently mixed, the OTR can be assumed to be identical to the oxygen uptake of the culture. In the Ames RAMOS test, the bacterial cells consume oxygen to maintain their metabolism and form new biomass. Thus, the OTR increases exponentially initially, while the histidine auxotrophic cells experience exponential growth on histidine. In Fig. 3, this effect is visible as an initial increase of the OTR over the first 4 h. At the end of this initial increase, the OTR reaches a peak of approximately 4 mmol/L/h. The supplied histidine is depleted at this point, and histidine-auxotrophic cells cannot grow any further. Since only maintenance respiration occurs, the OTR does not increase further. On the contrary, for the following 14–18 h, the OTR decreases due to histidine-auxotrophic cells lysing. During this time, histidine-prototrophic revertants can still grow and thus consume oxygen due to their biomass formation. However, their overall biomass is too low to be visible in the OTR since the OTR signal is comprised of the sum of histidine-auxotrophic and histidine-prototrophic (revertant) respiration. Only after about 18 h (PC) and 26 h (NC) the respiration activity of the revertants reaches a level where it impacts the OTR sum signal. For all PCs, the OTR reaches the threshold of 4 mmol/L/h at around 26 h, while the OTR of the NCs reaches the threshold about 30–31 h, which agrees with Kauffmann et al. (2020b).

According to a preliminary validation by Kauffmann et al. (2020b) and the mechanistic model mentioned above, we can assume that any

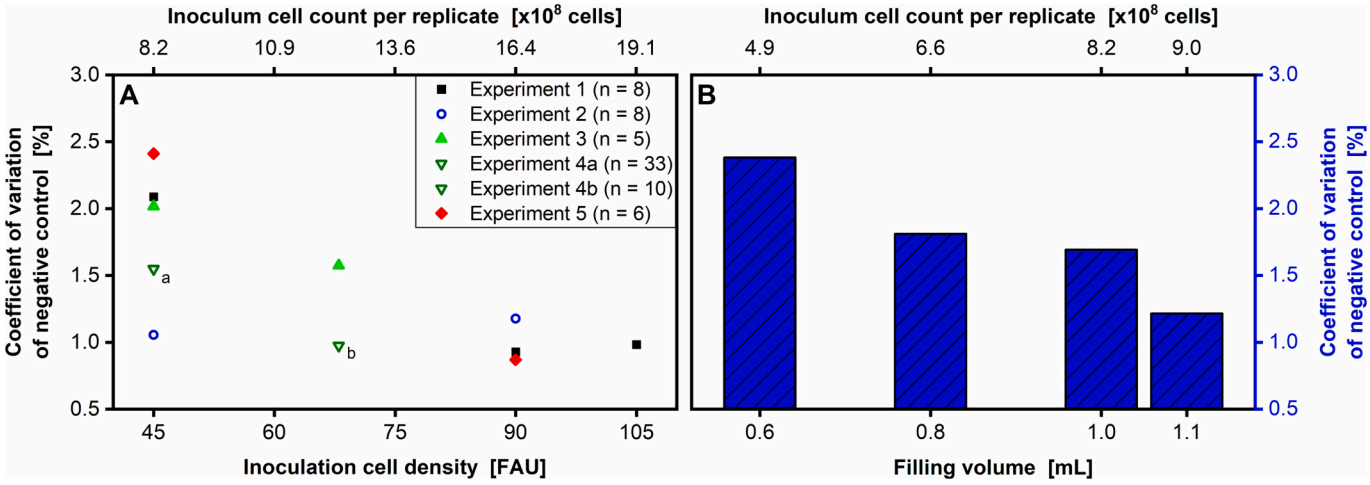


Fig. 5. Coefficient of variation (CV) for the negative control of *S. typhimurium* TA100 over different inoculum cell counts in 96-well scale. *S. typhimurium* TA100 in 1.2-fold concentrated optimized minimal medium containing 5 mg/L histidine (Section 2.2.3). (**A**) Inoculum cell count per replicate (upper x-axis) varied via the inoculation cell density in Formazine attenuation units (FAU) (lower x-axis) for a constant filling volume of 1 mL per well. Full data available in Figs. S7–S12 (Supplementary data). (**B**) Inoculum cell count per replicate (upper x-axis) varied via adjustment of filling volume per well (lower x-axis) for a constant inoculation cell density of 45 FAU.

soluble test substance will generate the same characteristic readout in all three scales of the Ames RAMOS test. Depending on a stronger or weaker mutagenic effect, the resulting OTR curves will be shifted along the x-axis (Kauffmann et al., 2020a, 2020b).

The NC's standard deviations (indicated as shadows in Fig. 3) increase with increasing throughput. While the OTR of NC exhibits a standard deviation of ±0.39 mmol/L/h at the OTR threshold in 48-well scale, the standard deviation almost doubles to ±0.80 mmol/L/h in 96-well scale. No such trend can be observed for the PC, where the standard deviation of the OTR at the OTR threshold is ±0.28 mmol/L/h and ± 0.26 mmol/L/h in 96-well scale.

3.3. Influence of initial cell count on the coefficient of variation (CV)

One main difference between the Ames RAMOS test scales is the filling volume per well. At the same optical density of 45 FAU, this results in different initial cell numbers per well subjected to the assay. By using a cell counter, the number of cells per mL was correlated to their optical density. Subsequently, the inoculum cell count per replicate (e.g., per well or plate) was determined for the experiments shown in Fig. 2 (Petri dish), Fig. 3 (48-well and 96-well MTP scale), as well as Fig. S3 (flask scale, Supplementary data). The results were compared to literature data from Pant et al. (2016). The results for the inoculum cell count per replicate and respective CV of the NC are shown in Fig. 4 (data from this work indicated by [a], data from Pant et al. (2016) indicated by [b]).

The Ames plate incorporation test with *S. typhimurium* TA100 (w/o S9) is usually conducted with approx. 10^8 cells per plate (Maron and Ames, 1983). Maron and Ames (1983) argue that the test results do not depend on the exact number of cells per plate, as their results for inoculum cell counts of 10^5 to 10^8 per plate were similar. However, the CV for these ranges was not compared. For the Ames plate incorporation test, literature reports CVs from 18 % (Pant et al., 2016), 16–21 % (Kato et al., 2018), and up to 26 % (Flamand et al., 2001). Pant et al. (2016) performed the Ames plate incorporation test in 6-well and 24-well format, using an inoculum cell count of 2×10^7 and 5×10^6 per well, respectively. For the 6-well format, the NC resulted in a CV of 23 %, while they reported a CV of 34 % for the NC in 24-well format (data indicated by [b] in Fig. 4). In this study (data indicated by [a] in Fig. 4), an inoculum cell count of 2×10^9 was used per Petri dish and a CV of 6 % derived, which is much lower than the literature values of 23 % and 34 % presented above (Pant et al., 2016). Both data from literature [b] and this work [a] presented in Fig. 4 indicate that a higher inoculum cell count per replicate correlates with a lower CV of the NC.

A similar trend was observed for the second test strain *S. typhimurium* TA98 (Fig. S4, Supplementary data), where we derived a CV of approx. 20 % with an inoculum cell count of 2×10^9 cells per plate. Pant et al. (2016), however, reported a CV >100 % for the Ames test in 24-well format with 5×10^6 cells per well. Overall, the CVs for the Ames RAMOS tests were much lower than those determined for the Ames plate incorporation tests, ranging from 0.3 to 2.5 %. Since our own experimental data and data reported in literature agree well for both *S. typhimurium* TA98 and TA100, we believe that our findings apply to all Ames test strains.

With 2×10^8 cells per well, the Ames RAMOS test in 48-well scale applies a similar cell count per replicate as the Ames plate incorporation test with 10^8 cells. For the NC in 48-well scale, a CV of 0.4 % was determined, while a CV of 18–26 % has been reported for the Ames plate incorporation test in literature. Since the cell broth in the submerged Ames RAMOS test is continually mixed, the cells can uniformly access nutrients and, thus, grow homogeneously. Agar cultures, however, are not mixed and grow more heterogeneously (see Section 3.1). Consequently, submerged and continually mixed test formats like the Ames RAMOS test result in an overall lower CV than solid agar cultivations.

These insights were further validated by varying the inoculum cell count in 96-well scale and observing the resulting CV of the NC. The inoculum cell count was varied in two ways: a) by adjusting the inoculum cell density to values between 45 and 105 FAU (Fig. 5A), and b) by changing the filling volume (Fig. 5B). In all cases, a higher inoculum cell count per replicate led to a lower CV of the NC. The same trend was observed in 48-well scale (see Fig. S5, Supplementary data).

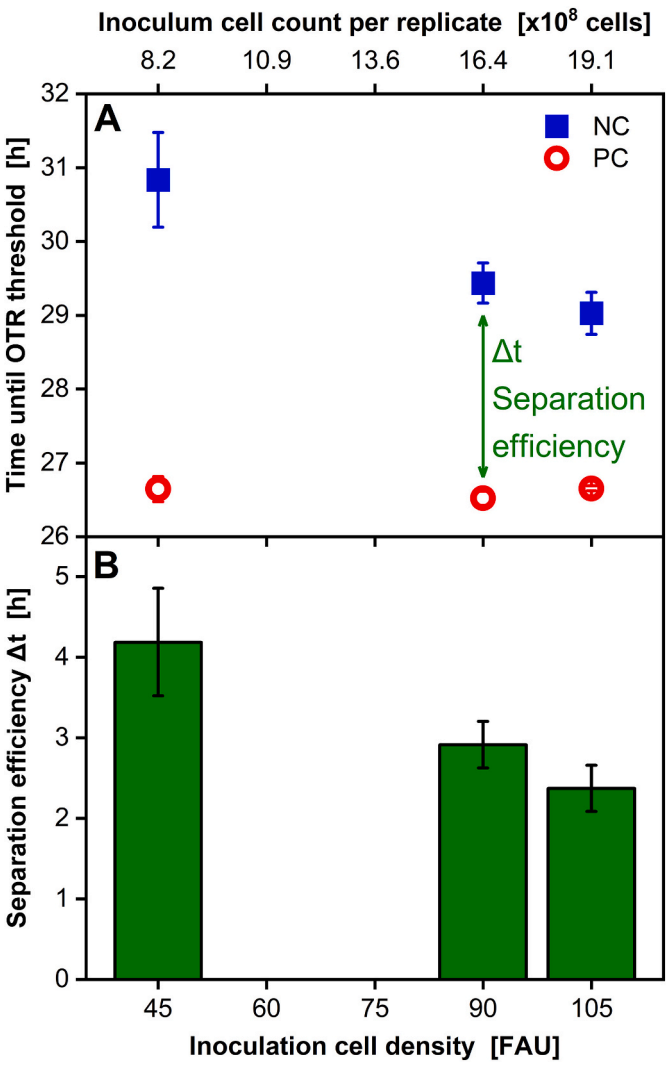
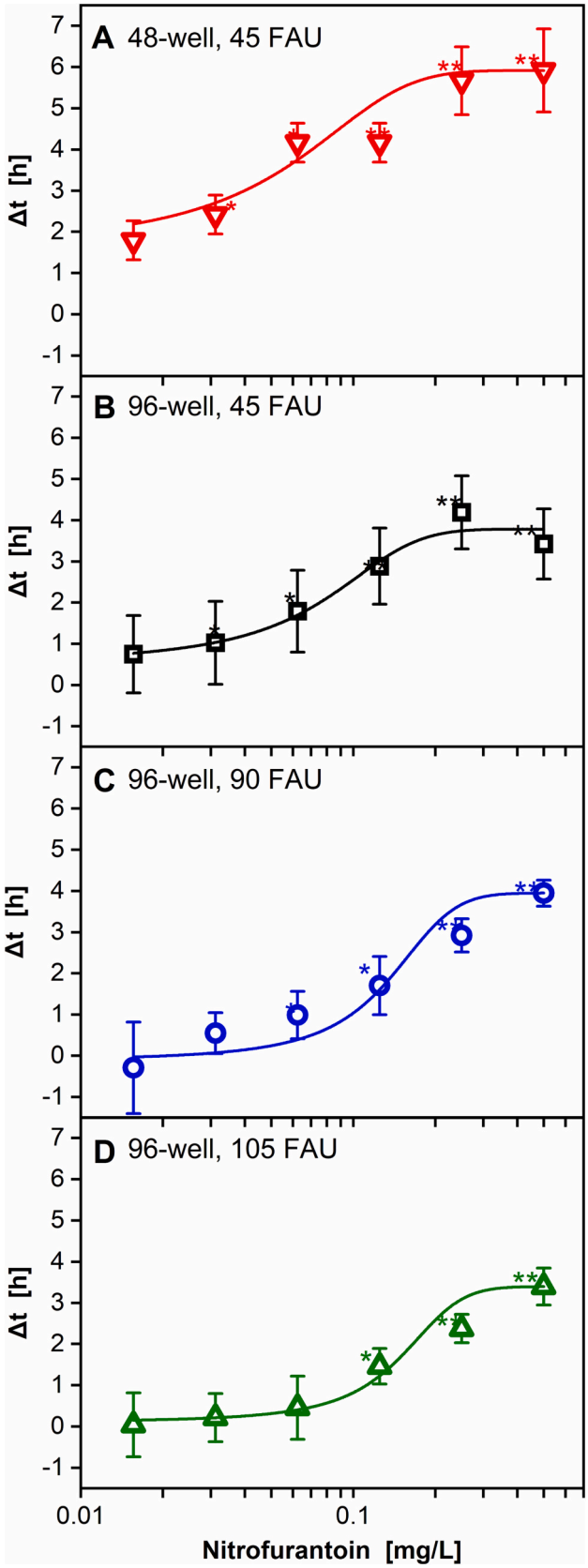


Fig. 6. Relationship between inoculation cell density and separation efficiency. *S. typhimurium* TA100 in 1.2-fold concentrated optimized minimal medium containing 5 mg/L histidine (see Section 2.2.3). (**A**) Comparison of negative control (NC, blue) and positive control (PC, red) readouts in 96-well scale. Variation of inoculation cell density in FAU (lower x-axis) at a constant filling volume of 1 mL per well. The corresponding inoculum cell count per replicate is shown on the upper x-axis. Readouts of time until OTR = 4 mmol/L/h in total cultivation hours, n = 8. (**B**) Separation efficiency Δt calculated according to Eq. (1). Full data in Fig. S8 (Supplementary data).

Generally, Ames test results are expected to follow a Poisson distribution (Margolin et al., 1981; Stead et al., 1981). When comparing experiments with different sample sizes or replicates, like in Fig. 5A, a lower CV is expected for a higher number of replicates according to the central limit theorem (Kwak and Kim, 2017). The central limit theorem states that the bigger the sample size, the narrower the distribution and the lower the standard deviation. Since this correlation could not be observed for the data in Fig. 5A, it is likely superimposed by another effect. Thus, we can assume that the exact replicate number is not causing the observed trend. Instead, the trend seems to originate with the sample taken from the culture broth for inoculation. The smaller the sample size and the lower the number of cells transferred, the broader the normal distribution of the occurring reverse mutations and the



(caption on next column)

Fig. 7. Dose-response curve results for nitrofurantoin from an Ames RAMOS test with *S. typhimurium* TA100. *S. typhimurium* TA100 in 1.2-fold concentrated optimized minimal medium containing 5 mg/L histidine (see Section 2.2.3). (**A**) 48-well scale: Initial cell density 45 ± 5 FAU, 2.4 mL filling volume, n = 6 replicates. This data was taken from Kauffmann et al. (2020b). (**B**) 96-well scale: Initial cell density 45 ± 5 FAU, n = 4 replicates. (**C**) 96-well scale: Initial cell density 90 ± 5 FAU, 1 mL filling volume, n = 4 replicates. (**D**) 96-well scale: Initial cell density 105 ± 5 FAU, 1 mL filling volume, n = 4 replicates. Separation efficiency Δt calculated according to Eq. (1). Statistical significance against negative control determined via two-sided *t*-test, * $p < 0.05$, ** $p < 0.01$. Full data in Fig. S15 (Supplementary data).

higher the CV of the NC. A similar trend was observed for the PC (Fig. S6, Supplementary data), although generally, the CV of the PC was much lower.

3.4. Influence of initial cell count on separation efficiency

While a low error of the NC is beneficial for detecting weak mutagens, the main variable to optimize is the separation efficiency. Kauffmann et al. (2020b) already investigated the influence of the initial histidine amount and cultivation temperature on the separation efficiency. Pant et al. (2016) compared NC (vehicle) and PC values for different formats of the Ames test but discussed neither the CV nor the separation efficiency between their controls. Thus, the influence of the inoculum cell count on the separation efficiency has yet to be addressed.

Fig. 6A shows the time until the OTR threshold was reached for PC and NC over three different inoculation cell densities, while Fig. 6B shows the resulting separation efficiencies. The highest separation efficiency of 4.1 h was observed for the lowest inoculation cell density of 45 FAU. Previous experiments in 48-well scale had shown that even lower inoculation cell densities did not increase the separation efficiency further (see. Fig. S13, Supplementary data). While the PC readout remains constant at 26.5 h for all three cell densities, a higher inoculating cell count correlates with an earlier readout for the NC. At an assumed constant back mutation rate, a higher inoculation cell density primarily results in a higher number of naturally occurring revertants. The higher number of revertants causes a head start on growth; consequently, the OTR threshold is read out earlier. However, for the PC, no such behavior could be observed. A possible reason for the constant PC readout over all three cell densities may be that the amount of nitrofurantoin per cell decreases with a constant nitrofurantoin concentration. If the mutation rate of the cells exposed to the PC is set by the natural back mutation rate plus the induced mutation rate through nitrofurantoin, then the latter could possibly decrease with a lower nitrofurantoin per cell ratio. Similar experiments were conducted for the test strain *S. typhimurium* TA98 (see Fig. S14, Supplementary data), but due to the high measurement error, no clear trend could be concluded.

Overall, doubling the cell number resulted in a roughly 25 % decrease in separation efficiency (Fig. 6B). As a lower CV of the PC and NC comes at the cost of the separation efficiency, the combined influence of CV and separation efficiency on detecting mutagenic substances was investigated through dose-response curves. Six nitrofurantoin concentrations were tested using each of the inoculum cell densities discussed above (Fig. 7B–D). In addition, a dose-response curve in 48-well scale previously published by Kauffmann et al. (2020b) is shown for reference in Fig. 7A.

All investigated inoculum cell densities revealed a dose-dependent increase of the separation efficiency and, thus, correctly classified nitrofurantoin as positive (mutagenic). However, the lowest detectable concentration of nitrofurantoin varied: In the 48-well scale reference, the second lowest nitrofurantoin concentration (0.03125 mg/L) was the lower limit of detection (LLOD). For an inoculation cell density of 45 FAU in 96-well scale, LLOD was also determined at the second-lowest dose. The LLOD for 90 FAU was found to be one dilution step higher at 0.0625 mg/L nitrofurantoin and another step higher at the fourth dose

(0.125 mg/L nitrofurantoin) for 105 FAU.

Rainer et al. (2021) recently published a direct comparison of LLOD for the Ames preincubation test and the Ames MPF. While nitrofurantoin was not investigated, the Ames MPF offered a five times lower LLOD for nine out of eleven substances than the Ames preincubation test. Proudlock and Evans (2016) had previously compared the LLOD of the Ames plate incorporation test and Ames test in 24-well scale (micro-Ames) for nitrofurantoin. The number of cells per mL of top agar was kept constant for their test protocol. Proudlock and Evans (2016) found that the LLOD of nitrofurantoin in the micro-Ames test was one dilution step lower than in the standard Ames plate incorporation test. Other substances, however, were detected at LLOD in the standard Ames plate incorporation test so that, overall, no clear trend could be formulated.

In this study, all tests succeeded in determining nitrofurantoin correctly as mutagenic. However, the trade-off between CV and separation efficiency at a higher inoculum cell density negatively influenced the lowest detectable test concentration. Thus, the inoculation cell density of 45 FAU seems best suited to ensure the correct determination even of weak mutagens.

4. Conclusion

This study measured for the first time the area-specific oxygen transfer (OTR′) of *S. typhimurium* TA100 during the Ames plate incorporation test. The OTR′ during the Ames plate incorporation test matched the shape of the OTR during the Ames RAMOS test, leading to the conclusion that the kinetic model introduced by Kauffmann et al. (2020a) applies to the Ames plate incorporation test as well. The translatability between the Ames plate incorporation and Ames RAMOS test was further demonstrated, as the evaluation of the Ames plate incorporation test via colony counts and through the OTR′ showed good agreement.

In the second part of this study, the lower inoculum cell count per replicate was identified as the primary variable responsible for a higher coefficient of variation (CV) in smaller scales for the Ames RAMOS and Ames plate incorporation tests. For both *S. typhimurium* TA98 and TA100, the CV was lower in the submerged and continuously mixed Ames RAMOS test compared to the Ames plate incorporation test. The effect of the inoculum cell count per replicate was further investigated, and its influence on separation efficiency was demonstrated as a higher inoculum cell count correlated with a lower separation efficiency between positive and negative control. The results of this study should be applied for a more rational, data-driven design of new Ames test protocols in small scale. Generally, the cell count and lower CV should be balanced against the separation efficiency to achieve an optimal lower limit of detection (LLOD), which is especially important when testing for weak mutagens that may be present in environmental samples.

Overall, the Ames RAMOS test offers several advantages when compared to established Ames test formats: First, the duration of the experimental protocol can be reduced by up to 50 %. Second, since data is recorded online, a manual evaluation of the results is no longer necessary. Third, deviations of test strain and medium quality become immediately apparent through the OTR signal, and operators can react in real time. In the future, the Ames RAMOS test needs to be validated with a range of chemical compounds and environmental water samples. In addition, the LLOD of the Ames RAMOS test should be compared against established Ames test formats in a larger study to examine whether the submerged, continually mixed Ames RAMOS protocol offers a more thorough detection of mutagens.

Funding

This work was funded by the DBU (Deutsche Bundesstiftung Umwelt, grant number AZ 34938).

CRediT authorship contribution statement

Eva Forsten: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Maurice Finger:** Methodology, Investigation, Formal analysis, Writing – review & editing. **Theresa Scholand:** Investigation. **Alexander Deitert:** Investigation. **Kira Kauffmann:** Methodology, Funding acquisition. **Jochen Büchs:** Resources, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank Philipp Wirtz for performing preliminary work in the laboratory, Kuhner Shaker GmbH for providing a μTOM prototype, Robert Dinger for helping with the μTOM device, as well as Andreas Schiwy for kindly providing the test strain *S. typhimurium* TA100.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.167035.

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