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Image-based quantification of fungal growth inhibition under ammonium fluoride exposure using MIPAR software: a preliminary proof-of-concept study

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ABSTRACT

This preliminary proof-of-concept study evaluated the feasibility of using MIPAR software for digital image-based quantification of fungal colony growth under ammonium fluoride (NH_4F) exposure. Growth dynamics of *Neurospora crassa*, *Aspergillus flavus*, and *Aspergillus niger* were monitored at 24, 48, 72, and 96 h using standardized digital imaging. Image segmentation enabled measurement of fungal colony surface area and visualization of temporal growth patterns. Under the experimental conditions investigated, NH_4F exposure was associated with substantial reductions in measurable colony area in *N. crassa* and *A. niger*, whereas *A. flavus* exhibited a more variable response. Calculated growth inhibition ranged from 98–100% for *N. crassa* and 71–94% for *A. niger*. For *A. flavus*, calculated inhibition was 58% at 24 h, whereas the treated colony area exceeded the control area at 48, 72, and 96 h. The findings demonstrate the feasibility and potential of MIPAR-based image analysis for objective monitoring of fungal colony growth under NH_4F exposure. Further studies incorporating independent biological replicates, appropriate controls, statistical analysis, and formal validation of image segmentation are required to establish the accuracy and reproducibility of the approach.

Keywords: fungal growth inhibition; ammonium fluoride; image analysis; MIPAR; fungal colony area; proof-of-concept

1. Introduction

Filamentous fungi such as *Aspergillus* spp. and *Neurospora crassa* are widely distributed microorganisms that play significant roles in food spoilage, biodeterioration, and the production of mycotoxins, posing both economic and health-related concerns (Xue et al., 2025; Kollath-Leiß et al., 2024). Among them, species of the genus *Aspergillus* are particularly relevant because of their rapid growth, environmental resilience, and ability to produce secondary metabolites under diverse conditions (Xue et al., 2025). In addition, *Neurospora crassa* is commonly used as a model organism in fungal physiology and growth studies (Kollath-Leiß et al., 2024).

Accurate quantification of fungal growth is important for evaluating antimicrobial effects and investigating fungal development dynamics. Conventional approaches, including manual measurement of colony diameter or visual scoring, are still widely used; however, they may be time-consuming and subject to operator-dependent variability, particularly when colony morphology is irregular or when colonies become dense and approach the boundaries of the available growth area (Hendricks et al., 2017).

Recent advances in digital image analysis have enabled the development of automated and more objective approaches for microbial quantification. Image-processing techniques can facilitate segmentation of fungal colonies from background surfaces and extraction of quantitative parameters such as colony area and growth kinetics (De Ulzurrun et al., 2015; Vismara et al., 2023; Zhang et al., 2026). Among these approaches, software-based solutions such as MIPAR provide flexible tools for image segmentation and quantitative image analysis and have been applied to two- and three-dimensional microstructural characterization (Sosa et al., 2014).

Digital image analysis therefore provides a complementary approach for quantitative assessment of fungal colony development by converting visual changes in colony coverage into measurable surface-area parameters. However, the reliability of such measurements depends on image acquisition conditions, colony morphology, segmentation quality, and appropriate spatial calibration. Filamentous fungi may exhibit irregular colony margins, heterogeneous pigmentation, and morphological changes during incubation, which can complicate accurate identification of colony boundaries. These factors should

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therefore be considered when applying image-based measurements to fungal growth assessment.

Fluoride compounds have been reported to affect microbial physiology through interactions with metabolic and cellular processes (Meiling et al., 2010; Li et al., 2012). Previous studies have also described effects of fluoride-containing compounds on fungal growth. Ammonium fluoride (NH_4F) was selected in the present study because of its high aqueous solubility and the relatively mild acidic-to-neutral reaction of its aqueous solutions, providing a suitable medium for evaluating fungal growth responses under fluoride exposure. However, because NH_4F contains both fluoride and ammonium ions, its biological effects cannot be attributed exclusively to fluoride without an appropriate ammonium control.

The application of automated image-based analysis to quantify the growth response of filamentous fungi exposed specifically to ammonium fluoride (NH_4F) remains insufficiently explored. This provides a basis for investigating whether digital image analysis can be used as a practical approach for monitoring changes in fungal colony area under NH_4F exposure.

In this preliminary proof-of-concept study, MIPAR-based image analysis was explored for quantitative assessment of fungal colony surface area following exposure to ammonium fluoride (NH_4F). Three filamentous fungi, *Neurospora crassa*, *Aspergillus flavus*, and *Aspergillus niger*, were monitored over a 96-h incubation period using standardized digital imaging. The study aimed to examine whether image-derived colony-area measurements could provide a practical quantitative descriptor of growth changes under NH_4F exposure and to identify methodological factors that may influence image-based assessment.

2. Materials and Methods

2.1. Fungal strains and antifungal assay

The antifungal activity of ammonium fluoride (NH_4F) was investigated using a modified poisoned-food technique (Balouiri et al., 2016). Three filamentous fungal species representing different experimental and environmental origins were selected: *Aspergillus niger* ATCC 16888, *Aspergillus flavus* (strain No. 4008/2, obtained from the collection of the Maize Research Institute Zemun Polje, Belgrade, Serbia), and *Neurospora crassa* (strain isolated from bread).

A. niger ATCC 16888 was included as a reference filamentous fungal strain, whereas *A. flavus* was selected as a relevant toxigenic *Aspergillus* species associated with agricultural and food-related environments. *N. crassa* was included as a filamentous fungal model with a different growth morphology from the *Aspergillus* species. The inclusion of these three organisms enabled comparison of image-derived colony-area changes under identical NH_4F exposure conditions across fungi with different biological and morphological characteristics.

Fungal cultures were grown on malt extract agar (LAB 37, Lab M, Bury, United Kingdom) at 28 °C for 24 h to allow spore germination. Mycelial plugs, 5 mm in diameter, were aseptically obtained using a sterile cork borer and placed upside down in the centre of Petri dishes containing the test medium.

For the NH_4F treatment, ammonium fluoride was incorporated into malt extract agar at a final concentration of 2 mg mL^{-1} prior to sterilization. Following sterilization, the pH of the medium was 5.4. Malt extract agar without NH_4F was used as the untreated control.

All inoculated Petri dishes were incubated at 28 °C, and colony development was monitored for 96 h. Digital images were acquired at 0, 24, 48, 72, and 96 h under standardized imaging conditions.

The experiment was conducted as a preliminary proof-of-concept experiment with one plate per experimental condition. Consequently, the measurements presented in this study represent individual plate measurements and were not used for formal statistical inference. The absence of independent biological replication is recognized as a limitation of the present study.

2.2. Digital image acquisition

Digital images of fungal colonies were acquired using an Olympus digital camera, model μ 1030 SW, with a 10.1-megapixel image sensor, 3.6× optical zoom, and an Olympus AF wide-angle zoom lens (5.0–18.2 mm, f/3.5–5.1).

Images were recorded at a resolution of 300 dpi, e.g., 3648 × 2736 pixels. The camera was positioned approximately 30 cm above the Petri dishes and oriented perpendicular to the sample surface. A camera flash was used as the illumination source during image acquisition. Photographs of the Petri dishes were taken using the camera's shooting-through-glass mode.

All images were acquired under the same imaging arrangement, including camera position, distance, orientation, and flash illumination, throughout the experiment. Petri dishes with a diameter of 60 mm were used. The imaging setup was kept unchanged between acquisition time points to minimize variations associated with illumination, camera position, and magnification.

Spatial calibration was performed in MIPAR using the known 60-mm diameter of the Petri dish as the reference dimension, allowing conversion of image dimensions from pixels to millimetres. The segmented fungal colony area was subsequently expressed in mm^2.

Because flash illumination occasionally produced local variations in image contrast, minor manual adjustments during image segmentation were required for some images. These adjustments were limited to the segmentation procedure and were applied to correctly identify the fungal colony region.

2.3. Image analysis using MIPAR software

Quantitative image analysis was performed using MIPAR software, version 4.50 (Sosa et al., 2014). The same general image-processing workflow was applied to all acquired images using a combination of automated segmentation operations and manual adjustments.

Each image contained two Petri dishes. First, the outer regions of the dishes were selected and inverted to separate the dishes from the background. The two dishes were then identified as separate layers. Within each dish, the uncolonized surface area was selected and inverted to segment the fungal colony into a separate layer. Variations in brightness and contrast caused by flash illumination occasionally interfered with segmentation and required image-specific manual corrections to the detected colony boundaries.

Accordingly, the procedure is considered semi-automated because manual corrections were required for some images. Although the overall processing workflow remained consistent, the extent of manual adjustment varied among images. Improvements in image acquisition may reduce the need for such corrections in future applications.

The workflow yielded four segmented layers per image, corresponding to the two Petri dishes and the two fungal colonies. Colony areas were calculated by MIPAR and converted to mm^2 using the spatial calibration based on the known Petri dish diameter.

A representative workflow is illustrated in Figure 1.

2.4. Calculation of growth inhibition

The apparent percentage of growth inhibition was calculated from the measured colony surface areas using the following equation:

$$I(\%) = 100 \times \frac{C - P}{C}$$

where C represents the measured colony surface area of the untreated

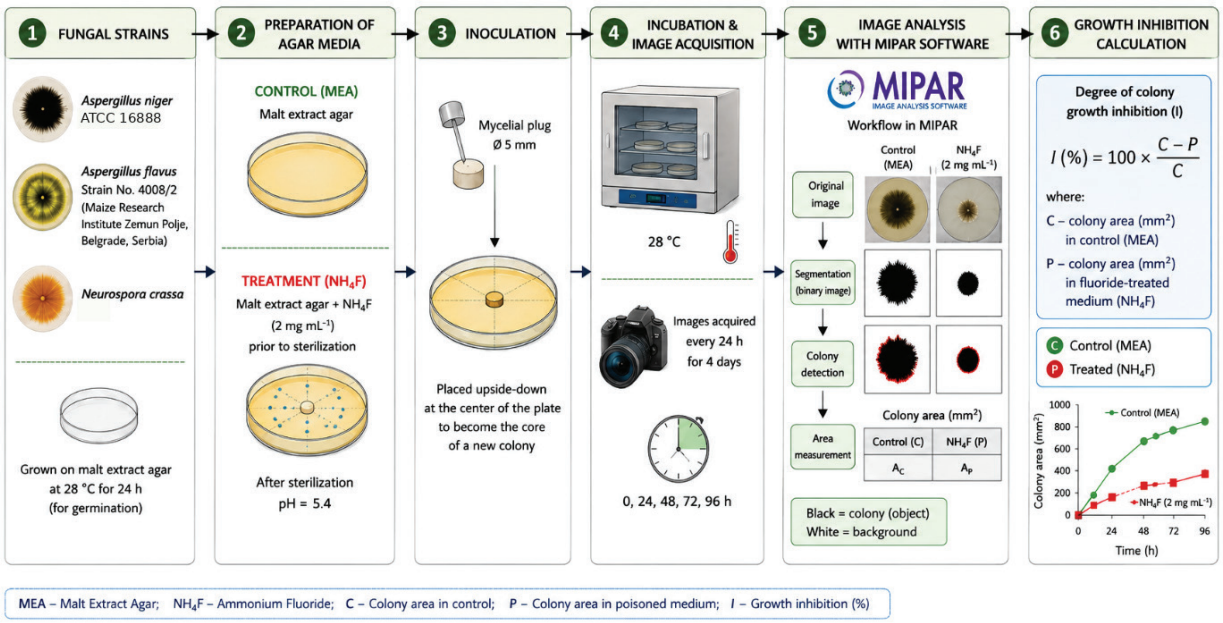


Fig. 1. Schematic workflow for image-based measurement of fungal colony area and quantification of ammonium fluoride-induced growth inhibition using MIPAR software.

control and *P* represents the measured colony surface area of the NH4F-treated sample at the corresponding incubation time.

Positive values indicate a smaller measured colony area in the NH4F-treated sample than in the corresponding control, whereas negative values indicate that the measured treated colony area exceeded the corresponding control area.

Because the experiment did not include independent biological replicates, the calculated inhibition values are presented as descriptive measurements rather than statistically validated estimates of treatment effect.

3. Results and Discussion

3.1. Digital monitoring of fungal colony development

Fungal cultures were cultivated on malt extract agar under control conditions and on medium supplemented with NH4F solution. Digital images were acquired at 24, 48, 72, and 96 h to monitor colony development.

The standardized imaging procedure enabled visual comparison of colony development between treated and untreated plates (Figure 2). Differences in colony expansion were apparent for all three fungal species, although the magnitude and temporal pattern of the observed response differed among species.

Image segmentation allowed the fungal colonies to be separated from the surrounding agar and converted into binary images for area measurement (Figure 3).

3.2. Quantitative image-based assessment of colony area

At 24 h, substantial differences in measured colony surface area were observed between NH4F-treated and control samples.

For *N. crassa*, the measured colony area was 41 mm² under NH4F exposure compared with 1717 mm² in the control, corresponding to a

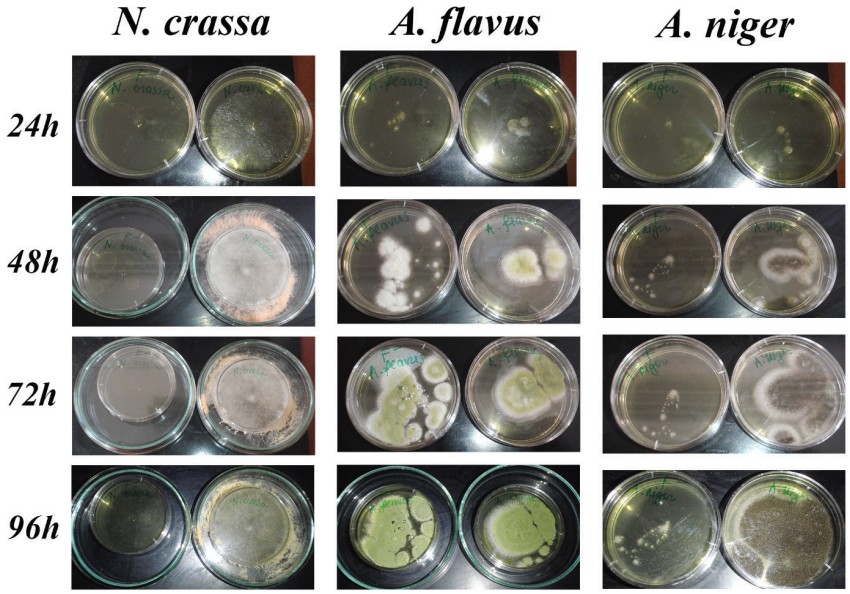


Fig. 2. Digital images of *Neurospora crassa*, *Aspergillus flavus*, and *Aspergillus niger* colonies after 24, 48, 72, and 96 h of incubation. Left plates correspond to NH4F-treated samples and right plates represent untreated controls. Images were acquired under standardized illumination and camera settings.

calculated inhibition of 98%. *A. niger* showed a treated colony area of 14 mm² compared with 49 mm² in the control, corresponding to 71% calculated inhibition. For *A. flavus*, the corresponding values were 44 and 105 mm², resulting in 58% calculated inhibition. These results indicate that NH4F exposure was associated with a marked reduction in measurable early mycelial expansion under the experimental conditions used.

At 48 h, no measurable colony area was detected for NH4F-treated *N. crassa*, while the control colony reached 2400 mm². The calculated inhibition was therefore 100%. *A. niger* showed a treated colony area of 81 mm² compared with 855 mm² in the control, corresponding to 91% calculated inhibition.

In contrast, the measured colony area of NH4F-treated *A. flavus* was 840 mm², compared with 767 mm² in the control. This resulted in a calculated inhibition of −10%. This value indicates that the measured colony area in the NH4F-treated plate exceeded that of the corresponding control at this time point. Because the experiment did not include independent biological replicates, this difference should be interpreted descriptively and may reflect biological variability, differences in colony morphology, or uncertainty associated with image segmentation.

At 72 h, *N. crassa* again showed no measurable colony area under NH4F exposure, while the control colony occupied the maximum measurable area of 2400 mm². *A. niger* showed a treated colony area of 98 mm² compared with 1721 mm² in the control, corresponding to 94% calculated inhibition.

For *A. flavus*, the measured treated colony area was 1482 mm² compared with 1186 mm² in the control, resulting in a calculated inhibition of −25%. This difference should be interpreted cautiously because the experiment did not include independent biological replicates and the measured area may have been affected by biological variability, colony morphology, and segmentation uncertainty.

At 96 h, *N. crassa* remained without measurable colony expansion in the treated condition, while the control remained at the maximum measurable area of 2400 mm². *A. niger* showed a treated colony area of 133 mm² compared with 2309 mm² in the control, corresponding to 94% calculated inhibition. For *A. flavus*, the treated and control areas were 2031 and 1992 mm², respectively, corresponding to −2% calculated inhibition.

The complete set of measured colony areas and calculated inhibition values is presented in Table 1.

3.3. Species-dependent response to NH4F exposure

The observed patterns indicate apparent differences in the response of the three fungal species to NH4F exposure. *N. crassa* exhibited the strongest apparent suppression of measurable colony expansion, with no measurable colony area detected after 48 h under the experimental conditions.

A. niger also showed a substantial reduction in measured colony area relative to the control, with calculated inhibition increasing from 71% at 24 h to 94% at 72 and 96 h.

In contrast, *A. flavus* showed a considerably more variable pattern. Although the calculated inhibition was 58% at 24 h, the measured treated colony area exceeded the control area at 48 and 72 h. Consequently, the calculated inhibition became negative. By 96 h, the difference between the treated and control colony areas was small.

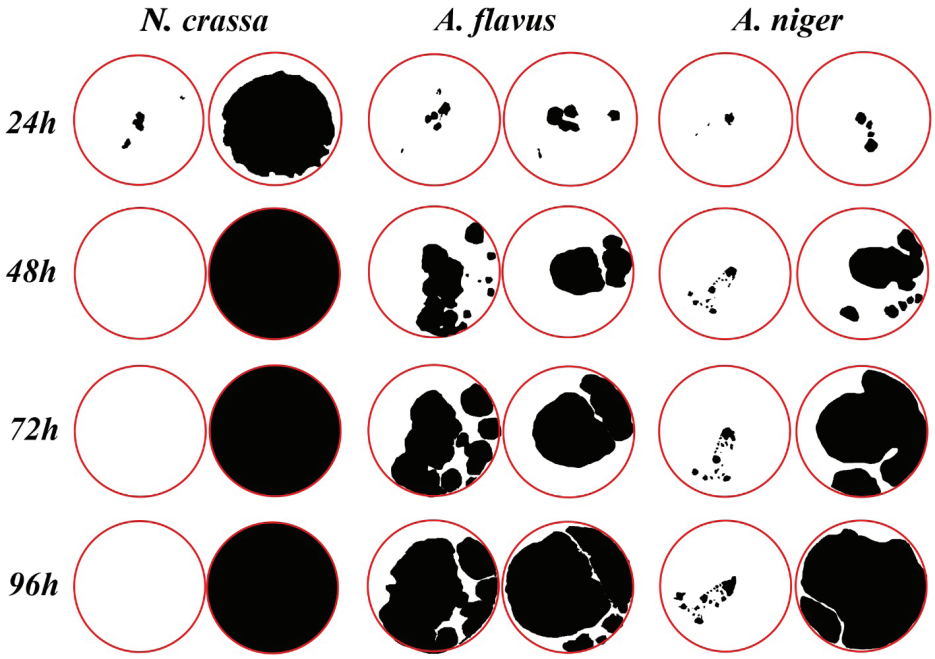


Fig. 3. Representative binary segmented images generated using MIPAR software. The detected fungal colony is represented as the black object of interest, whereas the surrounding uncolonized area is represented as the white background. Within each pair, the left image represents the NH4F-treated sample and the right image the untreated control. Images were acquired under standardized illumination and camera settings.

Table 1. Measured colony surface area and calculated growth inhibition of fungal species exposed to NH4F.

Incubation time	*N. crassa* T (mm²)	*N. crassa* C (mm²)	Inhibition (%)	*A. flavus* T (mm²)	*A. flavus* C (mm²)	Inhibition (%)	*A. niger* T (mm²)	*A. niger* C (mm²)	Inhibition (%)
24 h	41	1717	98	44	105	58	14	49	71
48 h	0	2400	100	840	767	−10*	81	855	91
72 h	0	2400	100	1482	1186	−25*	98	1721	94
96 h	0	2400	100	2031	1992	−2*	133	2309	94

*Negative calculated inhibition values indicate that the measured colony area in the NH4F-treated sample exceeded that of the corresponding untreated control. T = treated; C = control.

The negative inhibition values observed for *A. flavus* should not be interpreted as direct evidence of transient growth stimulation or physiological adaptation. Several factors could contribute to this observation, including natural biological variation, differences in colony morphology, heterogeneity of the fungal colony edge, and uncertainty associated with image segmentation. Because the present experiment did not include independent biological replicates, it is not possible to determine whether these differences represent a reproducible biological response.

The chemical composition of NH_4F should also be considered when interpreting the variable response of *A. flavus*. Ammonium fluoride provides both fluoride and ammonium ions, and therefore the observed response may reflect the combined effects of these species and the resulting chemical environment. The high aqueous solubility of NH_4F facilitates its use in aqueous fungal-growth experiments, while its solutions provide a relatively mild acidic-to-neutral environment under the conditions considered in this study. In contrast, sodium fluoride (NaF), another soluble fluoride salt, produces a different solution environment because of its weakly alkaline character.

The possible contribution of ammonium ions is also relevant. Previous work has shown that different inorganic nitrogen sources can produce different responses in *A. flavus*. For example, ammonium chloride and ammonium sulfate were reported not to stimulate mycelial growth and aflatoxin production to the same extent as ammonium nitrate, which was associated with higher mycelial growth and aflatoxin production (Wang et al., 2017). Other inorganic salts have also been reported to affect *A. flavus* colony growth (Shekhar et al., 2009). These observations support the need for caution when attributing the present *A. flavus* response exclusively to fluoride ions. However, because an ammonium-only control was not included in the present experiment, the relative contributions of F^- and NH_4^+ cannot be resolved from the current data.

The present data therefore demonstrate differences in fungal growth under NH_4F exposure, rather than establishing the specific contribution of F^- independently of NH_4^+.

3.4. Image segmentation and methodological considerations

The MIPAR workflow enabled fungal colony regions to be separated from the surrounding agar and provided direct measurements of the segmented colony area. This was particularly useful for colonies exhibiting irregular morphology, for which a single radial diameter measurement may not adequately represent the occupied surface area.

However, the present study was not designed as a formal validation of MIPAR against an established reference method. Therefore, the results should not be interpreted as demonstrating validated measurement accuracy or general reproducibility.

An additional limitation occurred for *N. crassa* controls at 48–96 h. The measured value of 2400 mm^2 remained constant because the colony had effectively occupied the maximum measurable region defined by the image-analysis procedure. Once the colony reached this limit, further radial expansion could no longer be represented by an increase in measured area. Thus, the apparent plateau does not necessarily indicate that fungal growth itself ceased.

This saturation of the measurable area illustrates an important consideration for digital image-based fungal growth analysis. For quantitative kinetic studies, image acquisition should ideally be performed at sufficiently short intervals or using an imaging field large enough to prevent colonies from reaching the limits of the predefined measurement region.

Overall, the present findings indicate that MIPAR can be used to obtain objective colony-area measurements under controlled imaging conditions. However, further methodological validation is required before the approach can be considered a fully validated quantitative assay for antifungal screening.

Conclusion

This preliminary proof-of-concept study demonstrates the feasibility of using MIPAR-based digital image analysis to quantify fungal colony surface area under NH_4F exposure. The approach enabled objective visualization and measurement of colony expansion over time and revealed apparent species-dependent differences in the response of *Neurospora crassa*, *Aspergillus flavus*, and *Aspergillus niger*.

Under the experimental conditions investigated, *N. crassa* showed the strongest apparent suppression of measurable growth, while *A. niger* exhibited substantial but incomplete inhibition. *A. flavus* showed a more variable response, including time points at which the measured treated colony area exceeded that of the corresponding control. These findings should be interpreted cautiously because the present study was not designed with independent biological replication or formal validation of the image-analysis procedure.

The results support the potential use of MIPAR as a practical image-based tool for monitoring fungal colony growth. Further studies incorporating independent biological replicates, appropriate ammonium controls, statistical analysis, and comparison with manual or other established quantitative methods are necessary to establish the accuracy, reproducibility, and broader applicability of this approach.

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