Journal of Microbiological Methods 206 (2023) 106679

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

# Differential toxicity of bioorthogonal non-canonical amino acids (BONCAT) in *Escherichia coli*

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ARTICLE INFO

*Keywords:*
BONCAT
Non-canonical amino acids
AHA
HPG
Toxicity

ABSTRACT

Single-cell methods allow studying the activity of single bacterial cells, potentially shedding light on regulatory mechanisms involved in services like biochemical cycling. Bioorthogonal non-canonical amino acid tagging (BONCAT) is a promising method for studying bacterial activity in natural communities, using the methionine analogues L-azidohomoalanine (AHA) and L-homopropargylglycine (HPG) to track protein production in single cells. Both AHA and HPG have been deemed non-toxic, but recent findings suggest that HPG affects bacterial metabolism. In this study we examined the effect of AHA and HPG on *Escherichia coli* with respect to acute toxicity and growth. *E. coli* exposed to 5.6–90 μM HPG showed no growth, and the growth rate was significantly reduced at >0.35 μM HPG, compared to the HPG-free control. In contrast, *E. coli* showed growth at concentrations up to 9 mM AHA. In assays where AHA or HPG were added during the exponential growth phase, the growth sustained but the growth rate was immediately reduced at the highest concentrations (90 μM HPG and 10 mM AHA). Prolonged incubations (20*h*) with apparently non-toxic concentrations suggest that the cells incorporating NCAAs fail to divide and do not contribute to the next generation resulting in the relative abundance of labelled cells to decrease over time. These results show that HPG and AHA have different impact on the growth of *E. coli*. Both concentration and incubation time affect the results and need to be considered when designing BONCAT experiments and evaluating results. Time course incubations are suggested as a possible way to obtain more reliable results.

## 1. Introduction

Bacterial activity in natural communities is crucial for biochemical cycling and ecosystem services. Resolving the bacterial activity to the single bacterial cell provides an opportunity to explore the mechanisms regulating activity in bacteria. Bioorthogonal non-canonical amino acid tagging (BONCAT) has shown promise as a fast and relatively inexpensive method to study bacterial in situ protein synthesis at a single-cell level. BONCAT has been applied to study bacterial activity distributions in soil (Couradeau et al., 2019), anaerobic (Hatzenpichler et al., 2014) and marine environments (Leizeaga et al., 2017; Samo et al., 2014) as well as in pulmonary bacteria (Valentini et al., 2020). One general principle of BONCAT is that the non-canonical amino acids (NCAA) are incorporated into proteins by translational activity, supposedly without disrupting the physiology or metabolism of the cell (Steward et al., 2020). "Bioorthogonal" is hence referring to non-interacting with cellular functions (Hatzenpichler et al., 2016).

The most used NCAAs in microbial studies are the methionine analogues L-azidohomoalanine (AHA) and L-homopropargylglycine (HPG) (Hatzenpichler et al., 2020). Currently, several protocols are used for BONCAT experiments (e.g. Leizeaga et al., 2017; Hatzenpichler and Orphan, 2015; Samo et al., 2014). Despite adjustments between protocols, the general procedure of BONCAT in microbial studies is the same: A sample containing living bacterial cells is incubated with a certain concentration of NCAA which are taken up by the cells' metabolic machinery and incorporated into polypeptides through translational activity. After incubation, the cells are fixed and then labelled with a fluorescent dye having azide or alkyne moieties that complement and react with the alkyne or azide moieties of the NCAA in a "click" reaction (Hatzenpichler et al., 2016). The labelling is specific, and the cells may be observed and enumerated using fluorescence microscopy (Hatzenpichler et al., 2014) or flow cytometry (Lindivat et al., 2020).

Although several studies have attempted to develop optimized protocols for the BONCAT method for microbial systems, the

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https://doi.org/10.1016/j.mimet.2023.106679
Received 30 November 2022; Received in revised form 24 January 2023; Accepted 25 January 2023
Available online 30 January 2023

recommendation is to optimize procedures for each system (Samo et al., 2014). During optimization of the BONCAT protocol for an experiment involving *E. coli*, we observed unexpected metabolic effects of the NCAAs used. While incubations with high concentrations (≥ 100 μM) of NCAAs for more than a few generations has been thought to affect bacterial growth rate (Hatzenpichler et al., 2020; Hatzenpichler et al., 2014), we found effects on bacterial growth at concentrations lower than previously reported (Valentini et al., 2020; Hatzenpichler et al., 2016). To scrutinize these findings, we studied the effects of a wide range of concentrations of AHA and HPG on growth of *E. coli*. In addition to acute effects, we also studied the consequences of prolonged incubations.

## 2. Materials and methods

### 2.1. Media and reagents

Minimal M9 liquid media (M9) was made from an autoclaved M9 salt solution (48 mM $Na_2HPO_4{\cdot}2H_2O$; 22 mM $KH_2PO_4$; 19 mM $NH_4Cl$; 8.6 mM NaCl) by adding 5.6 mM glucose, 2 mM $MgSO_4{\cdot}8\ H_2O$ and 0.1 mM $CaCl_2$ from sterile stocks solutions. The final pH of the M9 medium was 7.0 at room temperature. M9 agar media (M9 agar) was prepared with 11.2 mM glucose and 1.5% agar. Phosphate-buffered saline (PBS; 2.7 mM KCl, 1.8 mM $KH_2PO_4$, 10.1 mM $Na_2HPO_4{\cdot}2H_2O$, 137 mM NaCl, pH 7.4) was prepared in deionized water and sterile filtered (0.2 μm). All ingredients above, except $MgSO_4{\cdot}8\ H_2O$ (Merck, Darmstadt, Germany), were acquired from Sigma-Aldrich (Saint-Louis, Missouri, USA).

L-azidohomoalanine (AHA) (Cat# 1066) and L-homopropargylglycine (HPG) (Cat# 1067) were acquired from Click Chemistry Tools (Arizona, USA), and methionine (Met; J61904) was acquired from Alfa Aesar (Massachusetts, USA). Stock solutions (100 mM) of AHA, HPG and Met were prepared in milli-Q water and sterile filtered (0.2 μm).

### 2.2. Bacterial strain and glycerol stock production

The *E. coli* strain DSM 103246 was acquired from the German Collection of Microorganisms and Cell Cultures. Glycerol stocks were produced from one colony grown in M9 media at 37 °C to a cell density of approximately $8.0 \times 10^8$ CFU $mL^{-1}$, determined by plate counts. Autoclave-sterilized glycerol was added to a final concentration of 20% and mixed completely with the culture. The glycerol-culture mix was frozen at −80 °C in 500 μL aliquots. For each experiment, one aliquot was used and then discarded.

### 2.3. Growth inhibition experiments

Overnight *E. coli* cultures (10 mL M9 inoculated with 100 μL thawed glycerol stock) were diluted 1:10 in pre-warmed M9 and grown until mid-exponential phase, $OD_{600} = 0.2 \pm 0.01$, i.e. when the culture reached half the maximum OD they typically obtain in the M9 medium used. The cultures were then diluted 1:10 in M9 and used as inoculum (20 μL). For the assays we used flat-bottomed, non-treated 96-well plates (VWR European Art. No. 734–2781) filled with 180 μL of M9 supplemented with either AHA, HPG or methionine (Met). In AHA and HPG assays, the wells contained final concentrations of 0.2, 0.4, 0.7, 1.4, 2.8, 5.6, 11, 23, 45 and 90 μM. AHA assays were in addition performed with 35, 70, 140, 280, 560 μM and 1.1, 2.3, 4.5, 9 and 18 mM. Met was used as substrate control in all assays and in the same concentration as the NCAAs. All assays were run with four parallel wells. Growth controls were inoculated as above but in M9 medium without AHA, HPG and Met. In negative controls the inoculum was replaced by 20 μL sterile M9 medium. The two latter controls were run with eight parallels each. Each assay was run three times.

The initial cell density in the assays was about $1.6 \times 10^6$ CFU $mL^{-1}$. The plates were incubated at 37 °C in a Perkin Elmer Enspire™ 2300 Multilabel plate reader programmed to measure $OD_{600}$ every 20 min for 18 h.

Before use, the microwell plate lids were made hydrophobic with 0.05% Triton X-100 to avoid condensation (Brewster, 2003). As triton X-100 is a potential cell lysing agent (Cornett and Shockman, 1978), control plates without this treatment were incubated in parallel, but no negative effects were observed after 18 h.

### 2.4. Acute inhibition experiments

Exponentially growing *E. coli* cultures were prepared as above, diluted 1:100 in pre-warmed M9 medium and pipetted into 96-well flat-bottomed microwell plates (180 μL per well). One row of 8 wells was filled with sterile M9 as a control. The plates were incubated in the plate reader until $OD_{600}$ reached 0.104 ± 0.001 (approx. 300 min) which is equivalent to about $3.9 \times 10^7$ CFU $mL^{-1}$. HPG and Met were then added to final concentrations of 2.8, 5.6 and 90 μM; or AHA and Met to final concentrations of 2.8 and 90 μM and 10 mM to designated rows in the plate. Three parallel wells were used for each substrate and concentration. To keep the well volume constant, the same volume was removed as was added. Incubation in the plate was then resumed and continued for at least 18 h.

### 2.5. BONCAT staining and flow cytometry

Overnight *E. coli* cultures were diluted 1:10 in prewarmed M9 and incubated at 37 °C until $OD_{600} = 0.2$. The cultures were then diluted 1:100 in prewarmed M9 supplemented with 0.2–90 μM AHA or HPG and incubated at 37 °C for 30 min or overnight (20 h) and preserved with 0.9% formaldehyde solution (Sigma-Aldrich 47,608).

For BONCAT staining and flow cytometry, we used the protocol outlined earlier (Lindivat et al., 2020) with minor modifications. In short, the samples were centrifuged at 14000 ×*g* for 10 min in 2 mL tubes, supernatants were removed and replaced by PBS (pH 7.4).Freshly made sodium ascorbate (Merck A4034) and aminoguanidine hydrochloride (Sigma-Aldrich 396,494) was then added to final concentrations of 5 mM, followed by addition of a freshly made dye premix giving final concentrations of 120 μM $CuSO_4$, 570 μM THPTA (Sigma-Aldrich 762,342) and 5.7 μM AlexaFluor™ 647 alkyne or piconyl azide dye (Click Chemistry tools Catalog no 1301 and 1300). The samples were then incubated for 1 h in the dark before they were centrifuged at 14000 ×*g* for 10 min. Supernatants were removed and the cells resuspended in 700 μL 0.02% Tween Tris-EDTA buffer. Prior to flow cytometric analysis, the samples were diluted 1:10–1:1000 in Tris-EDTA buffer and stained for 10 min in the dark with 10 μL/mL SYBR green I ($10^{-4}$ final dilution of stock) (Thermo Fisher Scientific). The samples were analyzed using the Attune NxT Flow Cytometer (Thermo Fisher Scientific) using a blue laser (488 nm, 50 mW) and a red laser (638 nm, 100 mW) at dye-specific excitation/emission wavelengths: 590/30 nm for SYBR Green I and 670/14 for AlexaFluor™ 647 (AF647). The flow rate was set at 100 μL/min. Bacteria were detected through gating of SYBR green-stained cells and BONCAT-positive cells were detected based on AF647-fluorescence.

### 2.6. Data analysis

The plate reader output data file (.csv) was processed using a Python script, where $OD_{600}$ values and plate maps were organized into Excel data sheets. Both types of experiments were repeated three times and the results are reported as the mean. Data analysis was performed in Excel and growth rate was estimated from the early exponential growth phase (i.e. from when $OD_{600} > 0.025$ and the following 100 min) by linear regression of natural logarithm (ln) transformed growth curves. The effect of the different NCAA-treatments was tested using two-tailed Student's *t*-test in Excel.

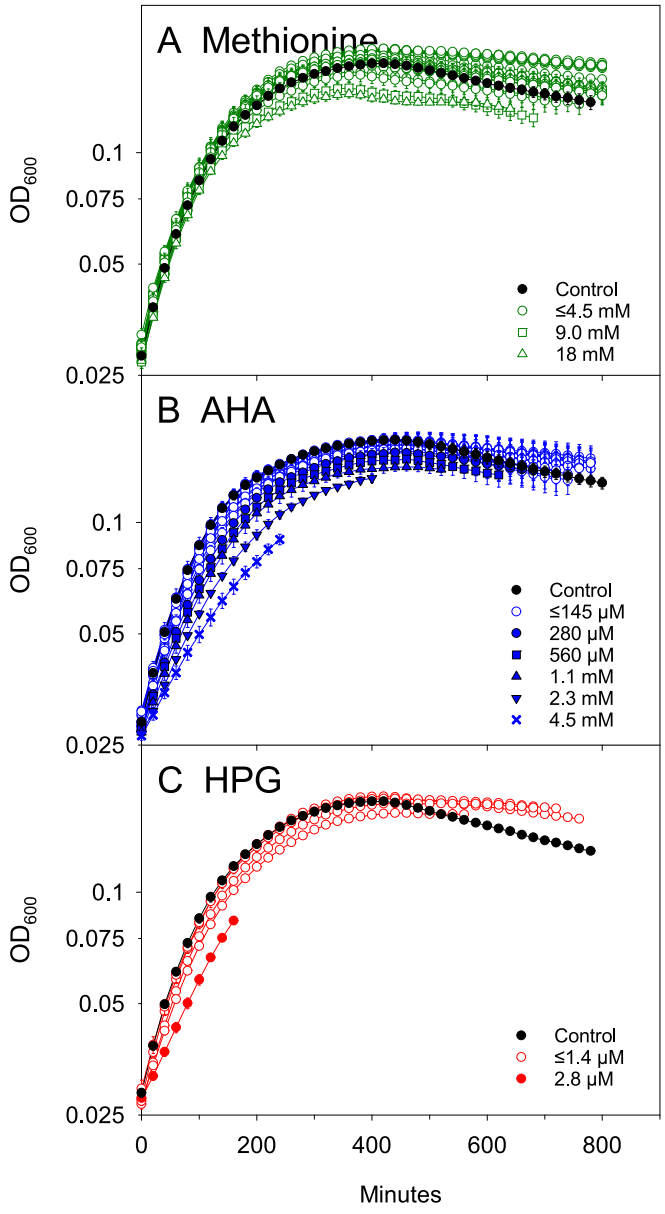


**Fig. 1.** Growth curves of *E. coli* in media containing different concentrations of methionine (A), L-azidohomoalanine (AHA, B), or L-homopropargylglycine (HPG, C). Each data point is the average of 3 independent experiments and error bars indicate standard error.

## 3. Results and discussion

Growth of *E. coli* at increasing concentrations of methionine, AHA and HPG compared to the respective control cultures (0 µM) is shown in Fig. 1. For Met we found no decrease in growth rate with increasing concentration (consider slope at origin), but the growth yield appeared to be lower at the two highest concentrations (Fig. 1 A). The growth rate decreased gradually at AHA concentrations between 280 µM and 4.5 mM (Fig. 1B). Lower concentrations (≤145 µM) had no significant effect (t-test, $p < 0.01$) while at the higher concentrations tested (9 and 18 mM) the cultures never exceeded $OD_{600} = 0.01$. We found significantly (*t*-test, $p < 0.01$) reduced growth rate at 2.8 µM HPG (Fig. 1C). At higher concentrations (5.6–90 µM), the cultures never exceeded $OD_{600} = 0.01$. Maximum growth rates ($\mu_{max}$) in the non-inhibited cultures were 0.63 ± 0.02 $h^{-1}$ (mean ± SD).

To investigate the acute effects, NCAAs and methionine were added in different concentrations to exponentially growing *E. coli* cultures

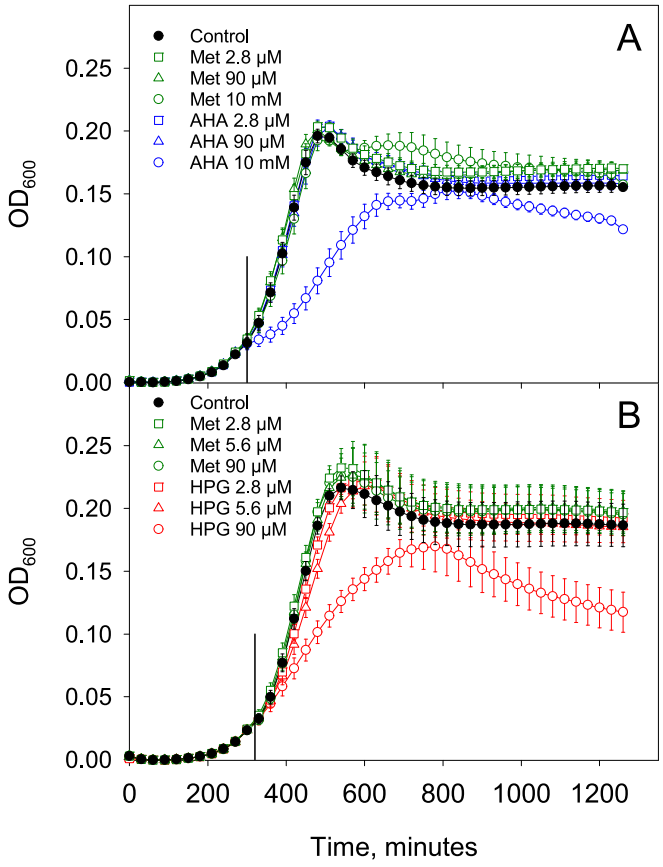


**Fig. 2.** Growth curves of *E. coli* in acute inhibition experiments where (A) HPG or Met and (B) AHA or Met were added in different concentrations during exponential growth. Addition of sterile M9 medium only served as control. Vertical lines indicates when AHA, HPG, Met or M9 (control) were added. Each data point is the average optical density ($OD_{600}$) from three independent experiments. Error bars represent the standard error. For clarity, only every third datapoint is plotted.

(Fig. 2). None of the tested HPG and AHA concentrations inhibited growth, but at the highest concentrations (90 µM HPG and 10 mM AHA) the growth rate was reduced immediately (0–90 min post addition, Student's t-test (two-tailed), $p < 0.02$ and $p < 0.001$ respectively) (Fig. 2). Met did not affect growth compared to the control culture at any concentration.

In summary, *E. coli* showed reduced growth when incubated with HPG concentrations >2.8 µM, but 90 µM was necessary to demonstrate an immediate effect on growth. AHA appeared to be less toxic, with reduced growth from >280 µM and > 9–10 mM for immediate impact on growth.

Previous studies have reported that AHA and HPG do not affect cell growth when low concentrations (≤ 50 µM) and short incubation times (≤1–2 generations) are used, but also that higher concentrations and/or longer incubation do have negative effects (Hatzenpichler et al., 2014; Pasulka et al., 2018). The concentrations of HPG we found toxic is lower than the 50 µM concentration frequently used and recommended in the literature (e.g. Hatzenpichler et al., 2016; Couradeau et al., 2019; Reichart et al., 2020; Steward et al., 2020) while the AHA concentrations used (50 µM – 6 mM; Wang et al., 2008; Steward et al., 2020; Valentini et al., 2020) are in the non-toxic range.

The negative effect of NCAA on cell growth and metabolism has been related to higher concentrations and longer incubation times, but as shown in the present study, the impact of both concentration and time is not straightforward. HPG appeared to have a negative effect on *E. coli* at a lower concentration than AHA. Wang et al. (2008) found only minor

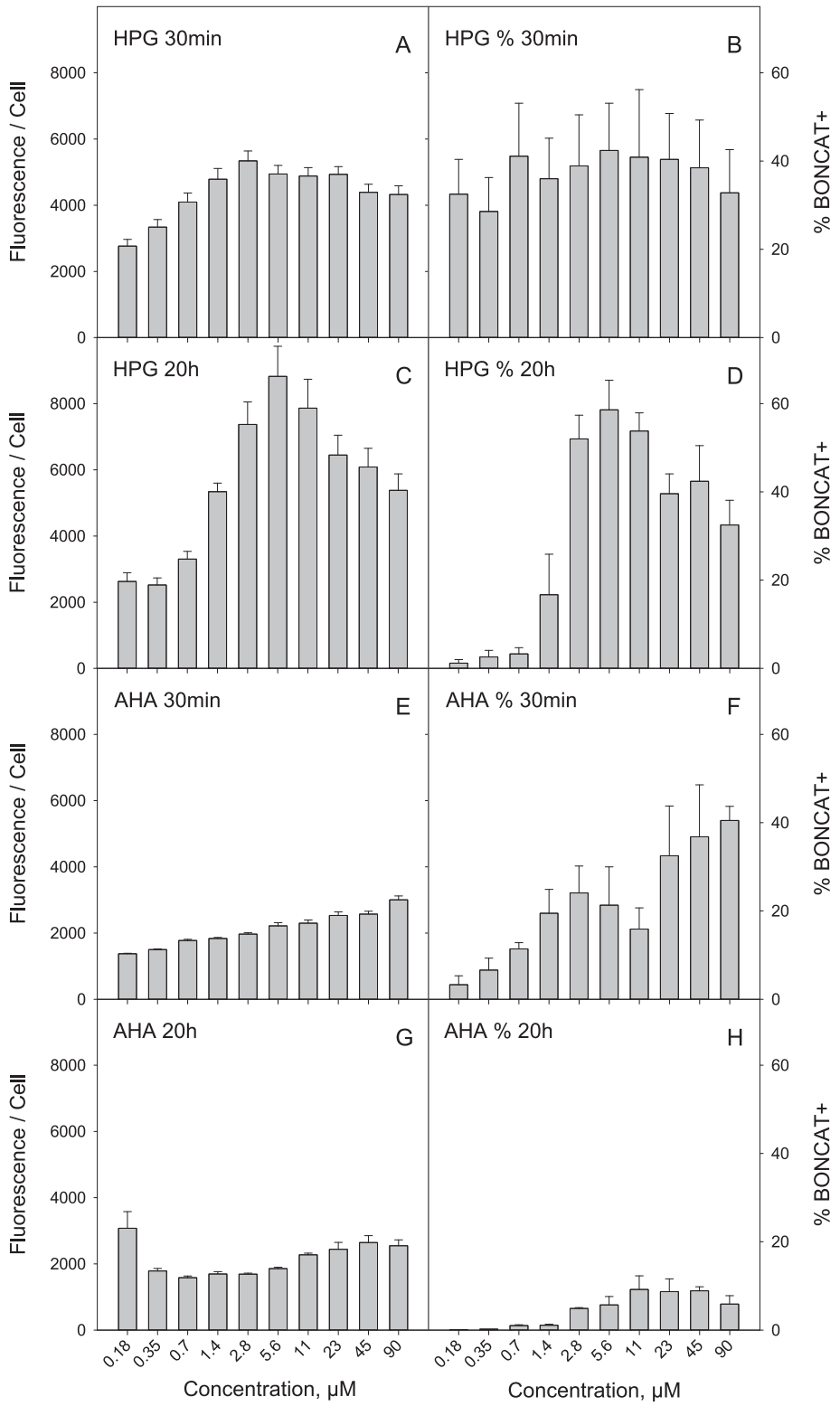


**Fig. 3.** Flow cytometric BONCAT data expressed as fluorescence/cell (left) and per cent BONCAT positive cells (% BONCAT +, right) for HPG (top half) and AHA (bottom half) with 30 min (A, B, E, F) and 20 h incubation times (C, D, G, H). X-axes show increasing concentrations of NCAA. Each bar is based on three replicate experiments with error bars showing standard error.

differences in metabolism of AHA and HPG in *E. coli*; both were efficient substrates for methionyl-tRNA synthetase and were efficiently incorporated into recombinant proteins with expression levels equivalent to those obtained with methionine. Nevertheless, differences in the NCAA uptake mechanism, which at present is unknown (Hatzenpichler et al., 2020), as well as differences related to misfolding and accumulation of non-functional NCAA labelled proteins (Hatzenpichler et al., 2014; Hatzenpichler et al., 2020) may at least to some extent contribute to explain the observed difference between HPG and AHA. In plants (e.g. *Arabidopsis thaliana*) AHA has been found to have a greater negative

impact on growth than HPG (Tivendale et al., 2021) and assessments of metabolic implications of NCAA should thus be considered for any target system as the effect appear inconsistent.

The flow cytometric BONCAT data show that *E. coli* take up HPG in increasing amounts up to growth inhibiting concentrations ($\geq$ 2.8 μM HPG) when incubated for 30 min, but also that the fraction of labelled cells was relatively even (37 $\pm$ 4%, mean $\pm$ SD) at all tested concentrations (Fig. 3A, B). When incubated for 20 h, however, both the amount of label per cell (Fig. 3 C) and the fraction of labelled cells (Fig. 3D) was reduced in the non-toxic range ($<$ 2.8 μM) while they remained high or increased in the toxic range ($\geq$ 2.8 μM HPG). In the same concentration range, where AHA appears to be non-toxic, both the uptake and the fraction of labelled cells increase with increasing AHA concentration (Fig 3 E, F). After 20 h incubation, the fraction of AHA labelled cells was reduced (Fig. 3 H), while the amount of label per cell remained unchanged or was slightly reduced (Fluorescence / Cell 30 min: 2110 $\pm$ 150; 20 h: 2160 $\pm$ 150 (mean $\pm$ SE, $n = 10$)) (Fig. 3 G), as was the case for HPG in the non-toxic range. This may suggest that the cells that incorporate NCAAs fail to divide and hence do not contribute to the next generation so that the relative abundance of labelled cells decrease. This implies that some cells, even in a single species population, grow but do not incorporate NCAAs, a hypothesis supported by increasing total cell counts in cultures with non-toxic HPG concentrations during the 20 h incubation. Alternatively, cells that remains metabolically active may after prolonged incubation somehow render the incorporated NCAAs unavailable for click reaction, while cells that are intoxicated by NCAAs remain unchanged and maintain the labelling obtained after short term incubation.

The growth inhibition and negative effects of NCAAs on cell metabolism may not be important for BONCAT studies as long as the initial rate of incorporation is related to growth and activity at the moment of NCAA addition and maintained long enough to be detectable. Care is nevertheless called for when interpreting uptake reported as fluorescence per cell or fraction of labelled (i.e. active) cells when based on a single, fixed incubation time. Results obtained from incubations lasting several generations (Fig. 3) appear to be upset by NCAA metabolism and such protocols need further testing and considerations for reliable interpretation.

Diversity in metabolism and potential species-specific response to NCAA amendments is another challenge that make the interpretations difficult when the BONCAT method is applied to mixed natural microbial communities (e.g., Hatzenpichler et al., 2016; Samo et al., 2014). Nonetheless, even for mixed communities, BONCAT provides an estimate of the number of protein-synthesizing cells and the signal intensity has been demonstrated to correlate with the rate of protein synthesis and is also used to assess the range of activity at the single-cell level (Samo et al., 2014; Leizeaga et al., 2017; Valentini et al., 2020). Flow cytometric fluorescence per cell data contains information both on the number of active cells and single cell activity (i.e., the intensity of the fluorescence signal is a proxy for single cell activity), whereas the fraction of active cells only carries the cell number signal. The fraction of active cells has the potential advantage that it only requires the synthesis rate in a cell to be maintained until the signal has reached detection level and may therefore be less sensitive to toxicity problems. The fluorescence per cell data should however provide more information on the underlying activity distribution and as a function of incubation time these data may be used to assess both growth rate and growth rate distribution. For incubations lasting several generations, assuming that non-functional proteins are not an issue, we may expect a decrease in per cell labelling rate because cells divide. The rate of cell labeling should decrease exponentially over time and fluorescence per cell at time $t$ can be expressed with the logarithmic equation $R_t = R_0(1 - 2^{-t/t_g})$; where $R_t$ = fluorescence per cell at time t, $R_0$ = fluorescence per cell in fully labelled cells, $t$ = incubation time, and $t_g$ = generation time. This equation implies that the growth rate may be estimated from time course incubations, providing a possible theoretical framework for interpretation of BONCAT data. Hence, when the basic limitations related to toxicity and inhibition vs incubation time are worked out and considered, we propose the BONCAT method may be more applicable than anticipated.

Further application and systematic experimentation on a range of different microbial systems, careful interpretation of results and data accumulation is the only way forward. In the meantime, we adhere to the general recommendation of keeping the NCAA concentrations low and incubation time to a minimum.

## Author contributions

SV provided funding. LL conceptualized the study with support of GB and SV. LL acquired data and performed analyses with support of GB and SV. JT produced software for data curation and analysis. GB created the figs. LL wrote the original draft and all authors contributed to reviewing and editing the paper.

## Declaration of Competing Interest

There are no competing interests.

## Data availability

Data will be made available on request.

## Acknowledgements

We thank T. Frede Thingstad for valuable insight regarding data interpretation, and Tatiana Tsagaraki for valuable input in the finalizing of this text. We also thank Elzbieta Petelenz for helpful discussions and assistance with FCM analyses. The work was supported by the Trond Mohn Research Foundation (TMS2018REK02).

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