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## Advanced spectroscopic analysis and <sup>15</sup>N-isotopic labelling study of nitrate and nitrite reduction to ammonia and nitrous oxide by *E. coli*†

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Nitrate and nitrite reduction to ammonia and nitrous oxide by anaerobic *E. coli* batch cultures is investigated by advanced spectroscopic analytical techniques with <sup>15</sup>N-isotopic labelling. Non-invasive, *in situ* analysis of the headspace is achieved using White cell FTIR and cavity-enhanced Raman (CERS) spectroscopies alongside liquid-phase Raman spectroscopy. For gas-phase analysis, White cell FTIR measures CO<sub>2</sub>, ethanol and N<sub>2</sub>O while CERS allows H<sub>2</sub>, N<sub>2</sub> and O<sub>2</sub> monitoring. The 6 m pathlength White cell affords trace gas detection of N<sub>2</sub>O with a noise equivalent detection limit of 60 nbar or 60 ppbv in 1 atm. Quantitative analysis is discussed for all four <sup>14</sup>N/<sup>15</sup>N-isotopomers of N<sub>2</sub>O. Monobasic and dibasic phosphates, acetate, formate, glucose and NO<sub>3</sub><sup>-</sup> concentrations are obtained by liquid-phase Raman spectroscopy, with a noise equivalent detection limit of 0.6 mM for NO<sub>3</sub><sup>-</sup> at 300 s integration time. Concentrations of the phosphate anions are used to calculate the pH *in situ* using a modified Henderson–Hasselbalch equation. NO<sub>2</sub><sup>-</sup> concentrations are determined by sampling for colorimetric analysis and NH<sub>4</sub><sup>+</sup> by basifying samples to release <sup>14</sup>N/<sup>15</sup>N-isotopomers of NH<sub>3</sub> for measurement in a second FTIR White cell. The reductions of <sup>15</sup>NO<sub>3</sub><sup>-</sup>, <sup>15</sup>NO<sub>2</sub><sup>-</sup>, and mixed <sup>15</sup>NO<sub>3</sub><sup>-</sup> and <sup>14</sup>NO<sub>2</sub><sup>-</sup> by anaerobic *E. coli* batch cultures are discussed. In a major pathway, NO<sub>3</sub><sup>-</sup> is reduced to NH<sub>4</sub><sup>+</sup> via NO<sub>2</sub><sup>-</sup>, with the bulk of NO<sub>2</sub><sup>-</sup> reduction occurring after NO<sub>3</sub><sup>-</sup> depletion. Using isotopically labelled <sup>15</sup>NO<sub>3</sub><sup>-</sup>, <sup>15</sup>NH<sub>4</sub><sup>+</sup> production is distinguished from background <sup>14</sup>NH<sub>4</sub><sup>+</sup> in the growth medium. In a minor pathway, NO<sub>2</sub><sup>-</sup> is reduced to N<sub>2</sub>O via the toxic radical NO. With excellent detection sensitivities, N<sub>2</sub>O serves as a monitor for trace NO<sub>2</sub><sup>-</sup> reduction, even when cells are predominantly reducing NO<sub>3</sub><sup>-</sup>. The analysis of N<sub>2</sub>O isotopomers reveals that for cultures supplemented with mixed <sup>15</sup>NO<sub>3</sub><sup>-</sup> and <sup>14</sup>NO<sub>2</sub><sup>-</sup> enzymatic activity to reduce <sup>14</sup>NO<sub>2</sub><sup>-</sup> occurs immediately, even before <sup>15</sup>NO<sub>3</sub><sup>-</sup> reduction begins. Optical density and pH measurements are discussed in the context of acetate, formate and CO<sub>2</sub> production. H<sub>2</sub> production is repressed by NO<sub>3</sub><sup>-</sup>; but in experiments with NO<sub>2</sub><sup>-</sup> supplementation only, CERS detects H<sub>2</sub> produced by formate disproportionation after NO<sub>2</sub><sup>-</sup> depletion.

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### 1. Introduction

In the absence of oxygen (O<sub>2</sub>), *Escherichia coli* (*E. coli*) can utilise alternative terminal electron acceptors for anaerobic

growth, such as nitrate (NO<sub>3</sub><sup>-</sup>) and nitrite (NO<sub>2</sub><sup>-</sup>). The sequence of reductions from NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup> to ammonia (NH<sub>3</sub>, NH<sub>4</sub><sup>+</sup> at physiological pH) is generally referred to as Dissimilatory Nitrate Reduction to Ammonia (DNRA).<sup>1</sup> The coupling of these reductions to the oxidation of organic substrates, such as formate, enables the generation of a proton gradient across the cytoplasmic membrane. DNRA is considerably more efficient for obtaining energy than the mixed acid fermentation pathways utilised when electron acceptors are unavailable. The expression of the respiratory NO<sub>3</sub><sup>-</sup> and NO<sub>2</sub><sup>-</sup> reductases is tightly controlled by FNR, an O<sub>2</sub> sensitive transcription factor, and NarXL/NarQP, both of which are two-component NO<sub>3</sub><sup>-</sup>/NO<sub>2</sub><sup>-</sup> sensitive regulatory systems.<sup>2,3</sup>

Although DNRA is the major NO<sub>3</sub><sup>-</sup> reduction pathway in *E. coli*, the bacterium also generates minor amounts of the toxic radical nitric oxide (NO) from NO<sub>2</sub><sup>-</sup> reduction. The low

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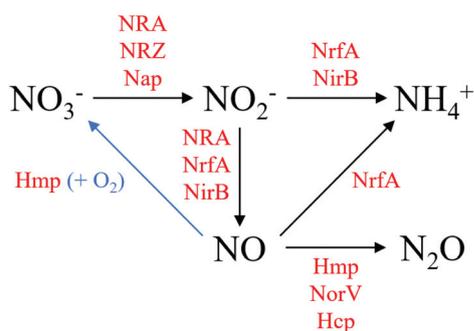
† Electronic supplementary information (ESI) available: S.1. Key nitrate and nitrite reduction enzymes, S.2. M9 medium formulation, S.3. FTIR spectroscopy of CO<sub>2</sub> and ethanol, S.4. cavity enhanced Raman spectroscopy (Experimental details, spectral fitting procedures and calibration plots), S.5. liquid phase Raman spectroscopy (Experimental details of the home-built Raman spectrometer, spectral fitting procedures and calibration plots) and S.6. analysis of bacterial culture samples (nitrite colorimetry, <sup>14</sup>N/<sup>15</sup>N-ammonium analysis). See DOI: 10.1039/d1an01261d



level of NO production by *E. coli* may be due to disproportionation of  $\text{NO}_2^-$  under acidic conditions or non-specific reduction by metalloproteins. The NADH-dependent cytoplasmic  $\text{NO}_2^-$  reductase (NirB),<sup>4</sup> the membrane-bound periplasmic  $\text{NO}_2^-$  reductase (NrfA)<sup>5</sup> and the major anaerobic  $\text{NO}_3^-$  reductase (NRA)<sup>6,7</sup> have all been proposed to be significant sources of NO formation as a by-product of their roles in the DNRA pathway. Aerobically, flavohemoglobin (Hmp) detoxifies NO by oxidation back to  $\text{NO}_3^-$ ; while anaerobically, NO is reduced further to nitrous oxide ( $\text{N}_2\text{O}$ ) reportedly by Hmp,<sup>8</sup> flavorubredoxin (NorV)<sup>9</sup> and hybrid cluster protein (Hcp).<sup>10</sup>  $\text{N}_2\text{O}$  is comparatively less toxic than NO and can rapidly diffuse out of the cell. *E. coli* is not a true denitrifier but  $\text{N}_2\text{O}$  production by  $\text{NO}_3^-$  respiring *E. coli* cultures does share similarities with the denitrification pathway of  $\text{NO}_3^-$  to nitrogen ( $\text{N}_2$ ) via  $\text{NO}_2^-$ , NO and  $\text{N}_2\text{O}$ . A summary of DNRA and NO generation and detoxification is shown in Fig. 1.

As a model organism, DNRA has been studied extensively in *E. coli*; however, comparatively less is known about the minor pathway leading to  $\text{N}_2\text{O}$  and how its generation differs between  $\text{NO}_3^-$  and  $\text{NO}_2^-$  respiring cultures. To gain a better mechanistic understanding, monitoring the key compounds and parameters of these processes is essential. Accurate and reliable analytical techniques are crucial for understanding cell biochemistry and pathway elucidation. This represents a challenge for analytical chemistry, requiring a combination of advanced analytical techniques.

Mass spectrometry and chromatographic techniques are widely applicable to the detection and quantification of a broad range of metabolites.<sup>11</sup> The tandem gas chromatography-mass spectrometry technique is considered the gold standard for the general analysis of volatile organic chemicals.<sup>12</sup> Despite this, these techniques are not readily applicable to rapid, online analysis either due to the need for sampling or for downstream chemical/physical processing before analysis can occur. Electrochemical sensors are widely used for monitoring pH, conductivity, dissolved  $\text{O}_2$  and various other chemical species,<sup>13</sup> including NO.<sup>14</sup> Often such sensors are suscep-



**Fig. 1** DNRA and NO generation and detoxification by *E. coli*. Enzymes are displayed in red: Hcp, hybrid cluster protein; Hmp, flavohemoglobin; Nap, periplasmic nitrate reductase; NirB, NADH-dependent nitrite reductase; NorV, flavorubredoxin; NRA, nitrate reductase A; NrfA, periplasmic nitrite reductase; NRZ, nitrate reductase Z.

tible to cross-interferences from other species, changes in solution activity and long-term drift. For microbiological studies, the need for physical contact between the electrode and cell culture increases the risk of contamination, particularly in continuous cultures, and requires that the electrode is stable towards sterilisation.<sup>11</sup>

Spectroscopic techniques can be readily applied for monitoring bioprocesses *in situ* and online, with no sampling. Vibrational spectroscopic techniques, such as Fourier Transform Infrared (FTIR) and Raman spectroscopies, show high specificity for different molecules due to characteristic spectral bands, making them potentially very valuable for metabolic studies. Additionally, vibrational spectroscopies can distinguish different isotopologues and isotopomers, allowing online monitoring of isotope labelling experiments.<sup>15,16</sup> Good sensitivities are observed in the condensed phase, but measuring headspace gases often suffers from low sensitivity, and special enhancement techniques are required such as Cavity Enhanced Raman Spectroscopy (CERS)<sup>15–22</sup> or long-path absorption White cells in FTIR spectroscopy.<sup>23</sup> Partial pressures in the headspace can be converted into concentrations in the solution *via* Henry's law. Quantum Cascade Laser (QCL) absorption spectroscopy has been applied to detect  $\text{N}_2\text{O}$  and other trace gases,<sup>24–26</sup> while sensitive, the limited tuning range of QCLs over a single IR absorption band limits the dynamic range due to band saturation effects. While FTIR spectroscopy has found some application in bioprocess monitoring, the broad absorption profile of water limits its application for monitoring metabolites at low concentrations in solution. In the gas-phase, the lack of an extended hydrogen network confines the absorption of water to certain spectral regions; molecules with absorption bands outside these regions can be readily detected, even in the presence of high levels of water vapour. Since Raman spectroscopy is comparatively insensitive to water, it is more readily applied to direct monitoring of the liquid-phase. However, fluorescence in complex media such as Lysogeny Broth (LB) can complicate the detection of the comparatively weak Raman light. Fluorescence can be avoided by moving to longer excitation wavelengths or by using media free of fluorescent components, such as M9 minimal media.<sup>23</sup> Vibrational spectroscopic tools have been previously applied to monitoring  $\text{NO}_3^-$  metabolism in bacteria; CERS has been used to follow  $\text{N}_2\text{O}$  and  $\text{N}_2$  production in denitrifying organisms, with the use of  $^{15}\text{NO}_3^-$  to produce  $^{15}\text{N}_2$  distinguishable from background  $^{14}\text{N}_2$ .<sup>19,22</sup> A robust CERS instrument has also been designed for field application to study the gas composition of soil samples.<sup>21</sup>

We report a combined approach for characterizing DNRA and  $\text{N}_2\text{O}$  production in anaerobic *E. coli* batch cultures using mostly non-invasive spectroscopic techniques. Sampling of the bacterial culture was only done for  $\text{NO}_2^-$  colorimetry and FTIR detection of  $^{14}\text{NH}_3$  and  $^{15}\text{NH}_3$  isotopomers. Headspace gas analysis was provided by the complementary techniques of FTIR and CERS, with CERS being a technique recently introduced by us in this Journal.<sup>17</sup> FTIR allowed detection of  $\text{CO}_2$ , ethanol and  $\text{N}_2\text{O}$  while CERS enabled monitoring of the homo-



nuclear diatomic molecules  $N_2$ ,  $O_2$  and  $H_2$ . Recently we introduced the capability of liquid culture analysis by Raman spectroscopy to monitor the microbial fermentation products of acetate and formate and the resulting *in situ* pH from phosphate signatures using a modified Henderson–Hasselbalch equation.<sup>23</sup> Here, we report on improvements that also allowed  $NO_3^-$  and glucose analysis during DNRA. With the use of  $^{15}N$ -labelling, we report on mechanistic insights into  $NO_3^-$  and  $NO_2^-$  reduction to  $NH_4^+$  and  $N_2O$  through interpreting the different  $^{14}N/^{15}N$ -isotopomers produced. The aims of this report are to introduce and characterise a unique combination of advanced spectroscopic techniques with great potential for bioanalytical applications, and to introduce an interesting biochemical application, a  $^{15}N$ -isotope labelling study on  $N_2O$  production during DNRA by *E. coli*, with a focus on the differences observed between  $NO_3^-$  and  $NO_2^-$  reduction.

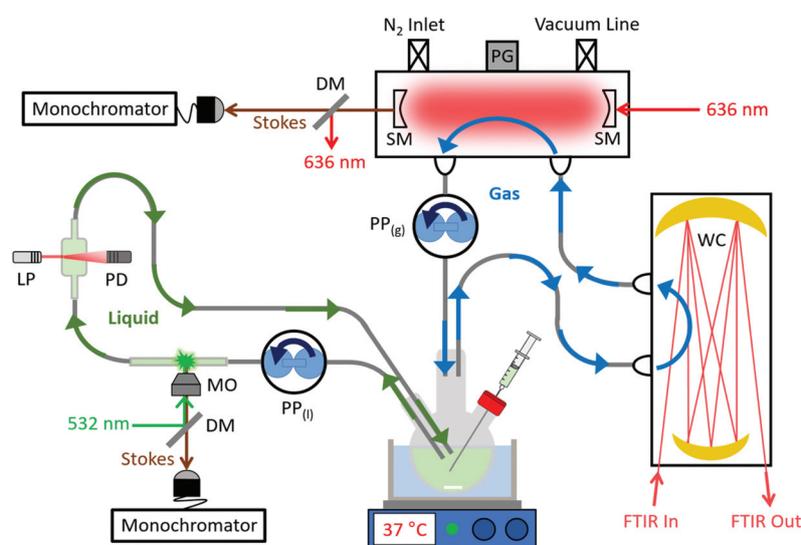
## 2. Experimental

Fig. 2 shows a scheme of our experimental setup. Since the previous iteration,<sup>23</sup> it was modified to include CERS for  $H_2$ ,  $N_2$  and  $O_2$  detection with larger headspace and culture volumes to compensate for more frequent sampling. 250 mL of bacterial batch culture is contained in a round bottom flask with two side-arm ports and submerged in a 37 °C thermostated water bath. From the left side-arm, the bacterial suspension is circulated using a peristaltic pump ( $PP_{(l)}$ , 4.5 L  $h^{-1}$ ) for *in situ*  $OD_{600}$  (optical density at 600 nm in a 1 cm cuvette) and Raman spectroscopy measurements. From the central-neck, the headspace (1425 mL volume) is cycled by a second peristaltic pump ( $PP_{(g)}$ , 4.5 L  $h^{-1}$ ) for gas-phase FTIR and CERS analysis. The right side-arm has a rubber septum enabling

sampling of the liquid culture for further analysis. The CERS cavity is equipped with a capacitance pressure gauge (PG),  $N_2$  inlet and vacuum line for purging  $O_2$  to give anaerobic growth conditions (1 atm  $N_2$ ) before starting experiments.

Production of  $CO_2$ , ethanol and  $N_2O$  was quantified by gas-phase FTIR spectroscopy (Mattson Research Series, 0.4  $cm^{-1}$  spectral resolution, MCT detector) with a home-built multiple-pass absorption White cell.<sup>23</sup> The White cell pathlength was adjustable between 4–8 m, with 6 m used for this work. Spectra were recorded every 5 minutes.  $CO_2$  partial pressures were obtained by integrating the  $\nu_1 + 2\nu_2 + \nu_3$  band (4920–5015  $cm^{-1}$ ,  $\nu_0 = 4978$   $cm^{-1}$ ) of the Fermi triad and comparing with a reference spectrum from the PNNL database.<sup>27</sup>  $N_2O$  partial pressures were obtained by integrating the  $2\nu_1$  combination band from 2460–2580  $cm^{-1}$  and comparing the integral with simulated spectra from HITRAN 2012.<sup>28</sup> All four  $^{14}N/^{15}N$ -isotopomers of  $N_2O$  could be distinguished, which enabled the  $^{15}N$ -isotope labelling studies. A multiplier equivalent to ethanol partial pressure was obtained by a least-squares fit of 1 ppmv ethanol and water reference spectra in the 2800–3100  $cm^{-1}$  region.<sup>23</sup> Using Henry's law, all partial pressures could be converted into concentrations in solution. Using the ideal gas law, we estimated that 10% of the  $CO_2$  present in the sample was dissolved. Under our conditions, less than 1% of dissolved  $CO_2$  was expected to be converted to carbonic acid and carbonates. 7% of  $N_2O$  and 99.7% of ethanol in the sample were also calculated to be dissolved.

The CERS setup has been described before with some modifications outlined below.<sup>15–17,20</sup> A 40 mW 636 nm single-mode cw-diode laser (HL63133DG) is coupled *via* a short-pass filter, a Faraday isolator and a mode matching lens into a linear optical cavity composed of two highly reflective mirrors (Newport SuperMirrors,  $R > 99.99\%$ ). If the laser wavelength

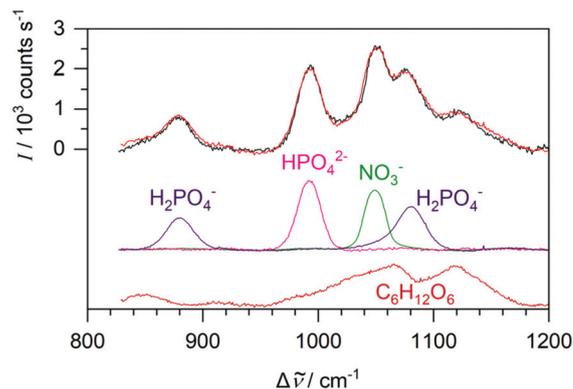


**Fig. 2** Experimental setup for analysing the headspace by CERS and White cell FTIR spectroscopies and the liquid culture by Raman spectroscopy and *in situ*  $OD_{600}$  measurements. DM, dichroic mirror; LP, laser pointer; MO, microscope objective; PD, photodiode; PG, pressure gauge;  $PP_{(g)}$ , gas-phase peristaltic pump;  $PP_{(l)}$ , liquid-phase peristaltic pump; SM, supermirror; WC, White cell.



matches the cavity length, an optical resonance builds up optical power inside the cavity by up to 3 orders of magnitude, enhancing the Raman signals. After the cavity, a dichroic mirror separates leftover excitation light from Raman signals which are coupled into a round-to-linear glass fibre bundle ( $7 \times \varnothing 105 \mu\text{m}$ ) and transferred to the monochromator (Andor Shamrock SR163,  $1200 \text{ l mm}^{-1}$  grating, DV420A-OE CCD). The  $400\text{--}2500 \text{ cm}^{-1}$  spectral range at  $6 \text{ cm}^{-1}$  resolution encompasses rotational S-branch lines of  $\text{H}_2$ , the  $\nu_1/2\nu_2$  Fermi resonance of  $\text{CO}_2$  and the vibrational fundamentals of  $\text{O}_2$  and  $\text{N}_2$ . Part of the leftover excitation light is diverted back to the diode for optical feedback, locking the laser to the cavity. To normalize Raman signals, the  $\text{N}_2$  peak is used as an internal standard since  $\text{N}_2$  is not expected to change during bacterial activity. Raman intensities are converted to partial pressures using tabulated integrated peak areas.<sup>20</sup>  $\text{CO}_2$  analysis by CERS was used to corroborate the FTIR analysis; however, CERS  $\text{CO}_2$  data was not displayed in this study due to FTIR  $\text{CO}_2$  detection being more sensitive. More details of the modified CERS setup are provided in the ESI.†

The bacterial suspension was circulated through a glass cuvette (1 cm path length) and the optical density  $\text{OD}_{600}$  was recorded *in situ* by measuring the scattering of red laser pointer light with a photodiode. The transmitted intensity was calibrated with start and end-point  $\text{OD}_{600}$  values externally measured using a UV-Vis spectrometer. The suspension was also circulated through a sealed borosilicate tube for recording liquid-phase Raman spectra using a home-built spectrometer.<sup>29,30</sup> A  $532.2 \text{ nm}$ ,  $20 \text{ mW}$  laser (Lasos, GL3dT) and monochromator (Shamrock SR-750-A,  $1200 \text{ l mm}^{-1}$  grating, DU420A-OE CCD) provided a spectral range from  $830\text{--}1710 \text{ cm}^{-1}$  at about  $0.8 \text{ cm}^{-1}$  resolution. Raman spectra were recorded every 5 minutes at 300 s integration time. No interfering fluorescence was noticeable in M9 minimal growth medium. The water bending vibration at  $1630 \text{ cm}^{-1}$  was used to normalise decreasing Raman intensities as the turbidity of the bacterial suspension increased.<sup>23</sup>  $0.1 \text{ M}$  reference spectra of individual glucose,  $\text{KNO}_3$ ,  $\text{CH}_3\text{CO}_2\text{NH}_4$ ,  $\text{HCO}_2\text{K}$ ,  $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$  solutions were recorded. As shown in Fig. 3, the  $830\text{--}1200 \text{ cm}^{-1}$  region contains characteristic Raman peaks for  $\text{HPO}_4^{2-}$  ( $989 \text{ cm}^{-1}$ ),  $\text{H}_2\text{PO}_4^-$  ( $876$  and  $1076 \text{ cm}^{-1}$ ),  $\text{NO}_3^-$  ( $1049 \text{ cm}^{-1}$ ) and glucose ( $960\text{--}1180 \text{ cm}^{-1}$ ).<sup>31</sup> Using a least-squares fitting routine, Raman spectra of the bacterial suspension in this region were fitted to the reference spectra, as well as a linear baseline. The returned multipliers of the reference spectra were then converted into concentrations *via* calibration plots. Noise analysis of background sample measurements (pure water) provided noise equivalent ( $1\sigma$ ) detection limits of  $0.6 \text{ mM}$   $\text{NO}_3^-$  and  $1.9 \text{ mM}$  glucose at 300 s integration time. With additional averaging to an integration time of 0.5 h (as was done with all time-dependent data displayed in this study), the limits improve to  $0.25 \text{ mM}$  for nitrate and  $0.8 \text{ mM}$  for glucose. The concentrations of the phosphate anions were used to calculate the pH *in situ* using a modified Henderson-Hasselbalch equation.<sup>23,32</sup> A least-squares fit determined acetate and formate concentrations in the  $1310\text{--}1450 \text{ cm}^{-1}$



**Fig. 3** In black, an experimental Raman spectrum of M9 medium supplemented with  $10 \text{ mM}$   $\text{KNO}_3$  and  $30 \text{ mM}$  glucose. In red, the sum of the fitted  $\text{NO}_3^-$ , glucose,  $\text{HPO}_4^{2-}$  ( $47 \text{ mM}$ ) and  $\text{H}_2\text{PO}_4^-$  ( $22 \text{ mM}$ ) models shown below the overlaid spectra.

region to the sum of acetate ( $1414 \text{ cm}^{-1}$ ) and formate ( $1349 \text{ cm}^{-1}$ ) models and a linear baseline, as shown in the ESI.† At 300 s integration time, the noise equivalent ( $1\sigma$ ) detection limits of acetate and formate were  $2.6 \text{ mM}$  and  $1.5 \text{ mM}$ , respectively. These limits improve to  $1.1 \text{ mM}$  and  $0.6 \text{ mM}$  with additional averaging to 0.5 h integration time. Although  $\text{NO}_2^-$  has a peak at  $1326 \text{ cm}^{-1}$ , the feature was too weak to be used in this study ( $1\sigma = 5.0 \text{ mM}$ ). Furthermore,  $\text{NH}_3/\text{NH}_4^+$  had no usable features within our spectral range.

*E. coli* (strain K-12 MG1655) was transferred from glycerol stock (maintained at  $-80 \text{ }^\circ\text{C}$ ) and streaked on LB-agar plates. Plates were left to grow overnight at  $37 \text{ }^\circ\text{C}$ . Before a measurement,  $50 \text{ mL}$  of sterile LB medium was inoculated with a single colony and incubated anaerobically in a sealed  $50 \text{ mL}$  centrifuge tube for 16 h ( $37 \text{ }^\circ\text{C}$ ,  $200 \text{ rpm}$ ) to a typical  $\text{OD}_{600}$  of 1.2. From the starter culture,  $20 \text{ mL}$  was centrifuged, and the pellet resuspended into  $20 \text{ mL}$  of fresh M9 minimal medium. Our M9 medium formulation is given in the ESI;† but notably, it contains  $30 \text{ mM}$  glucose and  $18 \text{ mM}$   $\text{NH}_4\text{Cl}$ . The M9 medium was supplemented with  $10 \text{ mM}$   $\text{K}^{15}\text{NO}_3$  ( $10 \text{ mM}$ ,  $98 \text{ atom } \% \text{ }^{15}\text{N}$ , Sigma-Aldrich) and/or  $5 \text{ mM}$   $\text{KNO}_2$  (either  $^{14}\text{N}$  or  $^{15}\text{N}$ ). A further  $230 \text{ mL}$  of M9 medium was prepared in the round bottom flask with two side-arms. The flask was pre-warmed and maintained at  $37 \text{ }^\circ\text{C}$  using a thermostated water bath under rapid stirring to enable efficient gas transfer. The  $20 \text{ mL}$  M9 medium containing *E. coli* was added to the  $230 \text{ mL}$  M9 medium in the flask, giving a typical starting  $\text{OD}_{600}$  of 0.1. The flask was then sealed and purged of  $\text{O}_2$  by alternating between evacuating the headspace and refilling with  $\text{N}_2$  at least five times. Experiments began once CERS measurements confirmed no  $\text{O}_2$  remained.

During experiments,  $1 \text{ mL}$  of the bacterial culture was sampled every 40 min and centrifuged. The supernatant was analysed using a colorimetric method to determine  $\text{NO}_2^-$  concentration based on the Griess test.<sup>33</sup> Our M9 media began with a typical pH of 6.9 and ended between 5.0–5.5 due to organic acid excretion. With a  $\text{pK}_a$  of 9.25,  $\text{NH}_3$  exists almost



entirely as  $\text{NH}_4^+$  at acidic pH. For  $^{14}\text{N}/^{15}\text{N}$ -analysis of  $\text{NH}_4^+$  samples, 2 mL 1 M NaOH was added to 0.6 mL of sample to release  $\text{NH}_3$ . The gas was analysed by a second FTIR setup (Bruker Alpha FTIR,  $0.8\text{ cm}^{-1}$  spectral resolution) with a home-built White cell (2.0 m pathlength). Spectra were recorded every 5 minutes with around 30 minutes needed before  $\text{NH}_3$  concentration peaked in the headspace. The basified solution was rapidly stirred and the 2 L headspace in the closed system was cycled between the sample flask and White cell using a peristaltic pump. The  $\nu_2$  band is the strongest in the FTIR spectrum of  $\text{NH}_3$  and can be used for  $^{14}\text{N}/^{15}\text{N}$ -analysis.<sup>34</sup> At the end of bacterial activity, the suspension was centrifuged, washed and dried to record the dry biomass (typically around 200 mg when corrected for sampling). For comparison with the *in situ* spectroscopic pH measurements, the pH of start and end-point samples was recorded externally using a Mettler Toledo SevenMulti pH meter. See the ESI† for further experimental spectra and calibration plots for all aforementioned analytical techniques.

### 3. Results and discussion

#### 3.1 FTIR spectroscopy of $\text{N}_2\text{O}$ and its $^{14}\text{N}/^{15}\text{N}$ -isotopomers

$\text{N}_2\text{O}$  has four  $^{14}\text{N}/^{15}\text{N}$ -isotopomers, *i.e.*,  $^{14}\text{N}_2\text{O}$ , the structural isomers  $^{14}\text{N}^{15}\text{NO}$  and  $^{15}\text{N}^{14}\text{NO}$ , and  $^{15}\text{N}_2\text{O}$ .  $\text{N}_2\text{O}$  is amenable to  $^{15}\text{N}$ -isotope labelling studies due to the low natural abundance of the  $^{15}\text{N}$ -isotope (0.37%). In the 2000–3000  $\text{cm}^{-1}$  spectral range, characteristic partially rotationally resolved bands of the  $\text{N}_2\text{O}$  isotopomers are available for FTIR analysis. Apart from *ca.* 2250–2400  $\text{cm}^{-1}$  which is saturated by  $\text{CO}_2$ , this region is free from significant spectral interferences. The HITRAN molecular database contains line lists for the three most abundant  $^{14}\text{N}/^{15}\text{N}$ -isotopomers, excluding  $^{15}\text{N}_2\text{O}$ .<sup>28</sup> A survey of HITRAN and our experimental spectra has shown that the following vibrational bands are available for quantitative analysis, including band position of  $^{14}\text{N}_2\text{O}$ , integrated absorption cross-sections  $G$  and peak absorbances  $A_{\text{peak}}$  (defined as  $\ln(I_0/I)$ ) under our experimental conditions for 1  $\mu\text{bar}$  (1 ppmv) at 6 m path length: the  $\nu_3$  fundamental near 2224  $\text{cm}^{-1}$  with  $G = 5.55 \times 10^{-17}\text{ cm}$  and  $A_{\text{peak}} \approx 0.023$  for rotational lines in the *P*- and *R*-branches, the  $2\nu_1$  overtone near 2563  $\text{cm}^{-1}$  with  $G = 1.33 \times 10^{-18}\text{ cm}$  and  $A_{\text{peak}} \approx 6 \times 10^{-4}$  for rotational features in the *P*- and *R*-branches, and the  $\nu_2 + \nu_3$  combination near 2798  $\text{cm}^{-1}$  with  $G = 9.0 \times 10^{-20}\text{ cm}$  and  $A_{\text{peak}} \approx 2.6 \times 10^{-4}$  of its *Q*-branch. Characteristic spectral shifts allow distinction of the isotopomers, while their  $G$  and  $A_{\text{peak}}$  values remain essentially the same. For accurate quantitative results,  $A_{\text{peak}}$  should not exceed unity. The dynamic range of the  $\nu_3$  fundamental thus extends from trace levels up to *ca.* 45  $\mu\text{bar}$   $\text{N}_2\text{O}$ , the  $2\nu_1$  overtone up to 1.7 mbar, and the  $\nu_2 + \nu_3$  combination up to 3.8 mbar. This range can be extended by reducing the absorption pathlength of the White cell.

Fig. 4 shows the  $\nu_3$  fundamental with distinct *P*- and *R*-branch features, with  $^{14}\text{N}_2\text{O}$  having its origin near 2224  $\text{cm}^{-1}$ . In a spectrum containing only  $^{14}\text{N}_2\text{O}$ , a least-

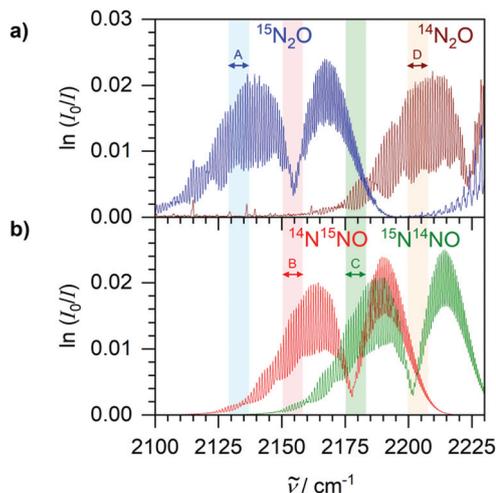
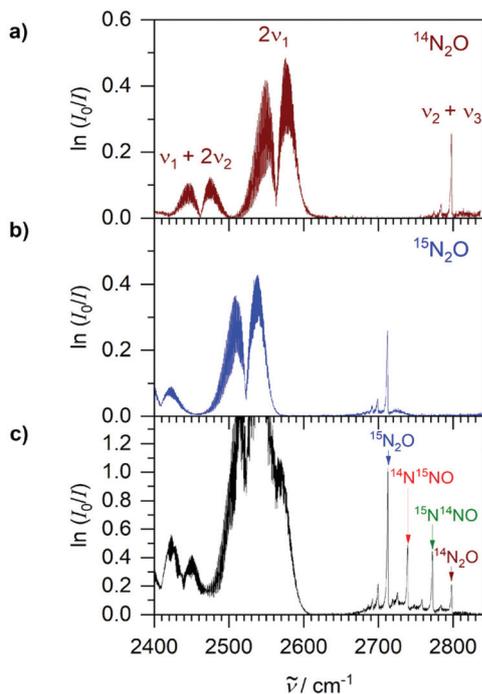


Fig. 4  $\nu_3$  fundamental of  $\text{N}_2\text{O}$  isotopomers with partially resolved rotational *P*- and *R*-branches. Absorbances scaled to correspond to 1  $\mu\text{bar}$  (1 ppmv) at 6 m path length. (a) Experimental FTIR spectra of  $^{15}\text{N}_2\text{O}$  (blue) and  $^{14}\text{N}_2\text{O}$  (brown). (b) Isotopomers (structural isomers)  $^{14}\text{N}^{15}\text{NO}$  (red) and  $^{15}\text{N}^{14}\text{NO}$  (green) calculated from the HITRAN database. A to D denotes spectral ranges used in the fit.

squares fit to the reference spectrum in the region denoted ‘D’ in Fig. 4 returns a multiplier which corresponds to  $\text{N}_2\text{O}$  partial pressure. A simple integration over the  $\nu_3$  band would not be suitable because part of the *R*-branch is buried in  $^{13}\text{CO}_2$  absorptions at higher wavenumbers. The region ‘D’ was selected because it has some of the strongest absorption features, it is very characteristic with partially resolved lines, and it is least affected by  $\text{CO}_2$ . With this fitting routine, noise analysis of blank samples provides a noise equivalent detection limit of 60 nbar (60 ppbv at 1 bar total pressure) at 6 m path-length and 128 accumulations which take 2 min to acquire. Detection limits can be improved by more averaging or increasing the path length. Note that this is sufficient to detect the 330 ppbv ambient levels of  $\text{N}_2\text{O}$  for environmental analytical applications. The heavier isotopomers shift to lower wavenumbers, 2201  $\text{cm}^{-1}$  for  $^{15}\text{N}^{14}\text{NO}$ , 2178  $\text{cm}^{-1}$  for  $^{14}\text{N}^{15}\text{NO}$ , and 2155  $\text{cm}^{-1}$  for  $^{15}\text{N}_2\text{O}$ . Since the bands are overlapping, only a simultaneous fit to all four model spectra can yield individual isotopomer partial pressures. A fit in the entire 2100–2220  $\text{cm}^{-1}$  region, however, has serious problems with cross-correlations. After a careful analysis, a simultaneous fit only including the regions ‘A’ to ‘D’ in Fig. 4 returned multipliers which are not noticeably affected by cross-correlations. Each region was chosen so that an individual isotopomer has a maximum weight with the other isotopomers having as little weight as possible. This procedure yields reliable isotopic partial pressures up to a dynamic range of about 45  $\mu\text{bar}$  per isotopomer.

Fig. 5 shows the weaker absorption bands that are more suitable for  $\text{N}_2\text{O}$  analysis above 45  $\mu\text{bar}$ . In isotopically pure samples, the  $2\nu_1$  overtone near 2563  $\text{cm}^{-1}$  can be integrated from 2505–2613  $\text{cm}^{-1}$  to obtain  $^{14}\text{N}_2\text{O}$  partial pressure after



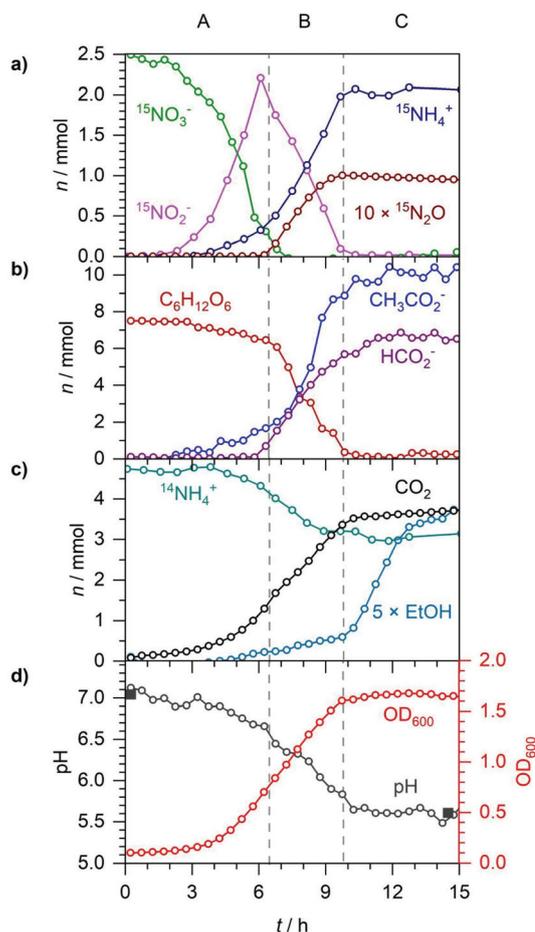


**Fig. 5** Experimental FTIR spectra of  $\text{N}_2\text{O}$  overtone and combination bands for (a) 1 mbar  $^{14}\text{N}_2\text{O}$ , (b) 1 mbar  $^{15}\text{N}_2\text{O}$  and (c) a mixture of 2.8 mbar  $^{15}\text{N}_2\text{O}$  (49%), 1.1 mbar  $^{14}\text{N}^{15}\text{NO}$  (20%), 1.2 mbar  $^{15}\text{N}^{14}\text{NO}$  (22%) and 0.5 mbar  $^{14}\text{N}_2\text{O}$  (9%). The isotopomer mixture was recorded at 30 h during the anaerobic respiration of *E. coli* supplemented with 10 mM  $^{15}\text{NO}_3^-$  and 5 mM  $^{14}\text{N}$ -nitrite (see section 3.4).

comparison with a reference spectrum (Fig. 5a). For  $^{15}\text{N}_2\text{O}$  the shifted band near 2523  $\text{cm}^{-1}$  can be integrated from 2460–2580  $\text{cm}^{-1}$  (Fig. 5b). In samples with mixtures of isotopomers (Fig. 5c), the  $2\nu_1$  bands overlap and require a more sophisticated simultaneous fit similar to the one described above for the  $\nu_3$  fundamental. Fortunately, this is not required as the  $\nu_2 + \nu_3$  combination band (2798  $\text{cm}^{-1}$  for  $^{14}\text{N}_2\text{O}$ ) has a sharp, characteristic Q-branch which remains well resolved and separated in isotopic mixtures. After comparison with reference spectra, simple integrations over the separate Q-branch peaks yield isotopic partial pressures in a mixture up to a dynamic range of about 3.8 mbar.

### 3.2 Spectroscopic analysis of nitrate reduction by *E. coli*

Fig. 6 is a typical example of pH,  $\text{OD}_{600}$  and number of moles ( $n$ ) of electron acceptors and other metabolites measured during the reduction of 10 mM  $^{15}\text{NO}_3^-$  by anaerobic *E. coli*. Concentrations (mM) in solution were converted to  $n$  (mmol) by multiplying by the culture volume (0.25 L), as were partial pressures using the ideal gas law ( $V = 1.425 \times 10^{-3} \text{ m}^3$ ,  $T = 310 \text{ K}$ ) and correcting for the dissolved percentage calculated *via* Henry's law. All biological experiments were repeated in triplicate, and all repeats showed essentially the same behaviour. The time-dependent data displayed in this study is for a single representative experiment selected from the repeats. Phase A (0–6.5 h) lasted until all  $\text{NO}_3^-$  was reduced to  $\text{NO}_2^-$ .



**Fig. 6** Anaerobic *E. coli* growth in M9 medium supplemented with 10 mM  $^{15}\text{NO}_3^-$ . A to C denotes three distinct phases:  $\text{NO}_3^-$  reduction (A),  $\text{NO}_2^-$  reduction (B) and  $\text{NO}_2^-$  depletion (C). (a) Time-dependent number of moles ( $n$ ) of  $^{15}\text{NO}_3^-$ ,  $^{15}\text{NO}_2^-$ ,  $^{15}\text{NH}_4^+$  and  $^{15}\text{N}_2\text{O}$  ( $\times 10$ ). (b)  $n$  of glucose, acetate and formate. (c)  $n$  of  $\text{CO}_2$ , ethanol ( $\times 5$ ) and  $^{14}\text{NH}_4^+$ . (d) Spectroscopically determined pH (open circles), externally measured pH (solid squares) and  $\text{OD}_{600}$ .

Phase B (6.5–10 h) lasted until all  $\text{NO}_2^-$  was reduced to  $\text{NH}_4^+$  and  $\text{N}_2\text{O}$ . Phase C (>10 h) had no electron acceptors remaining so the bacteria utilised fermentative pathways solely. The  $^{15}\text{N}$ -label transferred to  $^{15}\text{NH}_4^+$  and  $^{15}\text{N}_2\text{O}$  with no trace of other  $\text{N}_2\text{O}$  isotopomers formed. This was consistent with other studies that found the N-atoms in  $\text{N}_2\text{O}$  both originate from  $\text{NO}_3^-/\text{NO}_2^-$  and not other sources such as  $\text{N}_2$  or  $\text{NH}_4^+$ .<sup>19,22,35</sup> The externally measured start and end-point pH measurements showed good agreement with the time-dependent spectroscopically determined pH.

After a brief lag phase, exponential growth began at 3 h with a rapid increase in the  $\text{OD}_{600}$ .  $\text{NO}_3^-$  reduction to  $\text{NO}_2^-$  mirrored the growth curve with most of the  $\text{NO}_2^-$  produced excreted to prevent cytoplasmic toxicification.<sup>36</sup> *E. coli* expresses three  $\text{NO}_3^-$  reductases: the respiratory  $\text{NO}_3^-$  reductases A and Z (NRA and NRZ) and the periplasmic  $\text{NO}_3^-$  reductase (Nap).<sup>37–39</sup> NRA is the most active reductase at high  $\text{NO}_3^-$  levels (>2 mM).<sup>40</sup> Nap is induced by low  $\text{NO}_3^-$  levels, while



NRZ is expressed at low levels constitutively and may function under stress-associated conditions.<sup>40–42</sup>  $\text{NO}_2^-$  peaked at 2.2 mmol, less than the initial 2.5 mmol  $\text{NO}_3^-$ , as some  $\text{NO}_2^-$  was reduced alongside  $\text{NO}_3^-$  during A. 0.3 mmol  $^{15}\text{NH}_4^+$  and 1.6  $\mu\text{mol}$   $^{15}\text{N}_2\text{O}$  was produced, accounting for the total N-balance. Only 1% of the 0.3 mmol  $\text{NO}_2^-$  reduced in A was converted to  $\text{N}_2\text{O}$  instead of  $\text{NH}_4^+$ . *E. coli* expresses two  $\text{NO}_2^-$  reductases: the NADH-dependent cytoplasmic  $\text{NO}_2^-$  reductase (NirB) and the membrane-bound periplasmic  $\text{NO}_2^-$  reductase (NrfA). NirB likely produced  $\text{NH}_4^+$  during A as it is active when  $\text{NO}_3^-$  is readily available, unlike NrfA.<sup>43</sup> Evidence also suggests NirB can generate NO.<sup>4</sup> Anaerobically, NO is detoxified by reduction to  $\text{N}_2\text{O}$ , which is comparatively non-toxic and rapidly diffuses out of the cell. Flavorubredoxin (NorV),<sup>9</sup> hybrid cluster protein (Hcp),<sup>10</sup> NirB<sup>44</sup> and NrfA<sup>45</sup> have all been proposed to have NO detoxifying activity. Flavohemoglobin (Hmp) is primarily an NO oxidase but also acts as an NO reductase anaerobically.<sup>8</sup>

As *E. coli* does not possess any known  $\text{N}_2\text{O}$  reductases, further reduction to  $\text{N}_2$  was not expected. However, there is some evidence that  $\text{N}_2$  can be produced from high amounts of  $\text{N}_2\text{O}$  by a yet unknown mechanism.<sup>46</sup> To investigate whether under our conditions  $\text{N}_2$  was produced, we repeated the experiment, but under an argon atmosphere instead of  $\text{N}_2$ . No trace of  $\text{N}_2$  production was observed in the CERS spectra within our detection limit of ca. 0.2 mbar or 12  $\mu\text{mol}$   $\text{N}_2$ .

Formate oxidation to  $\text{CO}_2$  by the  $\text{NO}_3^-$ -inducible formate dehydrogenase (FdhN) is a physiological source of electrons for  $\text{NO}_3^-$  reduction.<sup>38</sup> Other sources include NADH, lactate and glycerol.<sup>37</sup> 1.7 of the 2.5 mmol  $\text{NO}_3^-$  reduced was coupled to FdhN activity as  $\text{CO}_2$  increased by such in A. The remaining 0.8 mmol  $\text{NO}_3^-$  was likely coupled to NADH oxidation.<sup>47</sup> As no formate was excreted in A, all formate produced by pyruvate formate lyase (PFL) must have been oxidised to  $\text{CO}_2$ . For each formate produced by PFL, one acetyl-CoA is formed which can be either directed into the anaerobic TCA cycle or converted to acetate (to produce ATP) or ethanol (to remove reducing equivalents). 1.7 mmol acetate and 0.05 mmol ethanol were excreted during A corresponding to 1.75 mmol formate, in good agreement with the 1.7 mmol  $\text{CO}_2$  produced. Acetate must be excreted to prevent cytoplasmic acidification and caused the extracellular pH to decrease from 7.1 to 6.7. The minor amount of ethanol produced was due to reducing equivalents being coupled directly into reduction of  $\text{NO}_3^-$ . Previous studies have found a similar repression of substrate-level NADH consuming pathways when electron acceptors are available.<sup>48</sup> Glucose decreased by 1.1 mmol owing to the production of  $\text{CO}_2$ , acetate, ethanol and biomass synthesis.

During phase B,  $\text{NO}_2^-$  was reimported into *E. coli* and reduced. From 6.5 to 10 h, 2.2 mmol  $^{15}\text{NO}_2^-$  was reduced almost linearly to 2.0 mmol  $^{15}\text{NH}_4^+$  and 0.1 mmol  $^{15}\text{N}_2\text{O}$ . 91%  $\text{NO}_2^-$  was reduced to  $\text{NH}_4^+$  and 9% to  $\text{N}_2\text{O}$ , a higher partitioning to  $\text{N}_2\text{O}$  than observed in A (1%). A higher partitioning to  $\text{N}_2\text{O}$  after  $\text{NO}_3^-$  was depleted is consistent with several studies of *E. coli* and *Salmonella enterica* that have implicated NRA as the enzyme that produces the majority of NO when  $\text{NO}_2^-$  is

abundant and  $\text{NO}_3^-$  absent.<sup>6,7,49,50</sup> NrfA, which is induced by  $\text{NO}_2^-$  but repressed by  $\text{NO}_3^-$ , may have also contributed towards the higher partitioning to  $\text{N}_2\text{O}$  in B as it has been proposed as a source of NO.<sup>5,51</sup> The radical NO has a distinct line-resolved absorption band centred at 1904  $\text{cm}^{-1}$  (for  $^{14}\text{NO}$ ) and a favourable partitioning into the headspace.<sup>52</sup> However, no intermediate  $^{15}\text{NO}$  gas was observed to accumulate, owing to its rapid detoxification to  $^{15}\text{N}_2\text{O}$  by *E. coli*. During B, a further 1.9 mmol  $\text{CO}_2$  was produced and the pH dropped from 6.7 to 5.7 due to the excretion of 5.7 mmol formate and a further 7.5 mmol acetate. Due to the 3 : 1 stoichiometry of formate oxidation to  $\text{CO}_2$ : $\text{NO}_2^-$  reduction to  $\text{NH}_4^+$ , 0.6 mmol  $\text{NO}_2^-$  was coupled to formate by NrfA.<sup>53</sup> The 5.7 mmol formate excreted during B would be plentiful to couple to the remaining 1.4 mmol  $\text{NO}_2^-$ . However, NrfA is most active at low  $\text{NO}_2^-$  levels while NirB is most active at high  $\text{NO}_2^-$  levels for detoxification of excess  $\text{NO}_2^-$ .<sup>36,43</sup> Thus, 1.4 mmol  $\text{NO}_2^-$  was likely reduced by NirB.

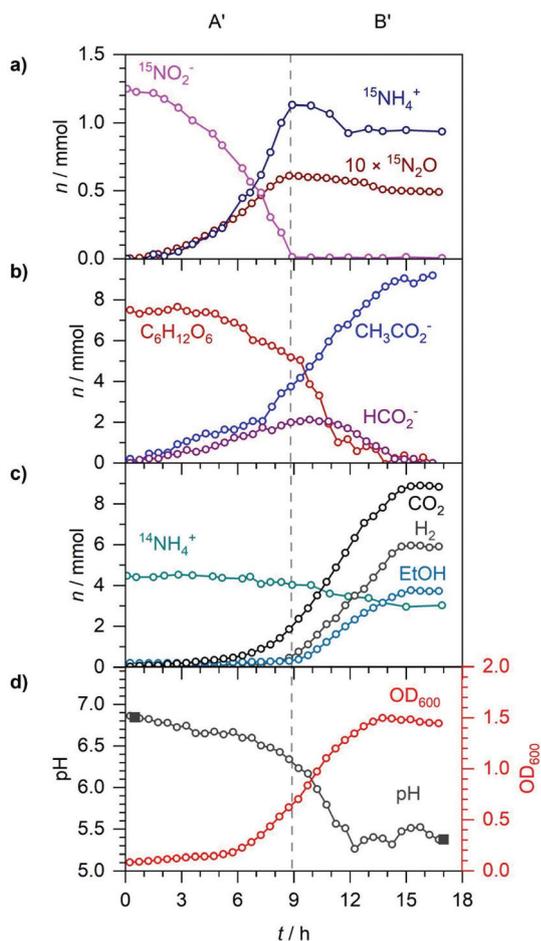
Phase C started with exponential growth ending as the  $\text{OD}_{600}$  peaked at 1.7, due to the depletion of glucose and  $\text{NO}_2^-$ . With no electron acceptors available, the bacteria funnelled reducing equivalents into ethanol as a further 0.7 mmol was made over the next 5 h. The remaining 5.7 mmol formate was slowly oxidised to  $\text{CO}_2$  at a rate of 0.03  $\text{mmol h}^{-1}$ . Under anaerobic conditions, the presence of formate induces formate hydrogenlyase (FHL) activity that disproportionates formate to  $\text{CO}_2$  and  $\text{H}_2$ .<sup>54</sup>  $\text{O}_2$  and  $\text{NO}_3^-$  repress FHL expression and instead induce the aerobic and the formate- $\text{NO}_3^-$  respiratory chains. High formate concentrations can partially reverse the repression by  $\text{NO}_3^-$ , but not by  $\text{O}_2$ .<sup>55,56</sup> However, CERS measurements detected no  $\text{H}_2$  production during our 10 mM  $^{15}\text{NO}_3^-$  reduction experiments. During C, there was a slight decline in  $\text{N}_2\text{O}$  observed due to the gas adsorbing to tubing and glass surfaces.

Experiments were terminated after 2 days with 5 mmol formate still remaining. The dry biomass was typically around 200 mg. As *E. coli* can be approximated to be 48% carbon and 14% nitrogen by mass,<sup>57</sup> ca. 8 mmol C and 2 mmol N in the biomass originated from the 7.5 mmol glucose (45 mmol C) and  $\text{NH}_4^+$ , respectively. 44 out of the 45 mmol C from glucose can be accounted for in the biomass, 5 mmol  $\text{CO}_2$ , 5 mmol formate, 12 mmol acetate (24 mmol C) and 1 mmol ethanol (2 mmol C). During exponential growth,  $^{14}\text{NH}_4^+$  decreased from 4.5 to 3.0 mmol accounting for 1.5 out of the 2 mmol N in the biomass. The remaining 0.5 mmol N likely was taken from the excreted  $^{15}\text{NH}_4^+$ . The 2.5 mmol  $^{15}\text{N}$ -label can be accounted for in the 2.0 mmol  $^{15}\text{NH}_4^+$ , 0.1 mmol  $^{15}\text{N}_2\text{O}$  (0.2 mmol  $^{15}\text{N}$ ) and ~0.5 mmol  $^{15}\text{NH}_4^+$  used for biosynthesis.

### 3.3 Spectroscopic analysis of nitrite reduction by *E. coli*

To study the response to  $\text{NO}_2^-$  alone, anaerobic *E. coli* was supplemented with 5 mM  $^{15}\text{NO}_2^-$ , as shown in Fig. 7. Phase A' (0–9 h) corresponded to the reduction of  $\text{NO}_2^-$  to  $\text{NH}_4^+$  with concurrent  $\text{N}_2\text{O}$  production via NO. Phase B' (9–15 h) was when the bacteria utilised fermentative pathways only, due to  $\text{NO}_2^-$  depletion. During the first 9 h of phase A', 1.25 mmol





**Fig. 7** Anaerobic *E. coli* growth in M9 medium supplemented with 5 mM  $^{15}\text{NO}_2^-$ . A' and B' denote two distinct phases:  $\text{NO}_2^-$  reduction (A') and  $\text{NO}_2^-$  depletion (B'). (a) Time-dependent number of moles ( $n$ ) of  $^{15}\text{NO}_2^-$ ,  $^{15}\text{NH}_4^+$  and  $^{15}\text{N}_2\text{O}$  ( $\times 10$ ). (b)  $n$  of glucose, acetate and formate. (c)  $n$  of  $\text{CO}_2$ ,  $\text{H}_2$ , ethanol and  $^{14}\text{NH}_4^+$ . (d) Spectroscopically determined pH (open circles), externally measured pH (solid squares) and  $\text{OD}_{600}$ .

$^{15}\text{NO}_2^-$  was reduced almost exponentially to 1.15 mmol  $^{15}\text{NH}_4^+$  (90%) and 0.06 mmol  $^{15}\text{N}_2\text{O}$  (10%), mirroring the bacterial growth curve which increased to an  $\text{OD}_{600}$  of 0.7. The 10% partitioning to  $\text{N}_2\text{O}$  here was consistent with the 9% observed during the  $\text{NO}_2^-$  reduction phase B in section 3.2. During A', 1.9 mmol  $\text{CO}_2$ , 3.7 mmol acetate, 2.0 mmol formate and 0.3 mmol ethanol were produced as glucose decreased from 7.5 to 5.2 mmol. Excretion of acetate and formate caused the pH to decrease from 6.9 to 6.3. The sum of acetate and ethanol (4.0 mmol) showed good agreement with the sum of  $\text{CO}_2$  and formate (3.9 mmol). Due to the 3:1 stoichiometry of formate oxidation:  $\text{NO}_2^-$  reduction and 1.9 mmol  $\text{CO}_2$  being produced, 0.63 out of the initial 1.15 mmol  $\text{NO}_2^-$  reduced to  $\text{NH}_4^+$  was coupled to formate oxidation to  $\text{CO}_2$ . The remaining 0.52 mmol  $\text{NO}_2^-$  was likely reduced *via* coupling to NADH oxidation by NirB.

Phase B' began at 9 h when  $^{15}\text{NO}_2^-$  was depleted. *E. coli* could only utilise fermentative pathways in the absence of

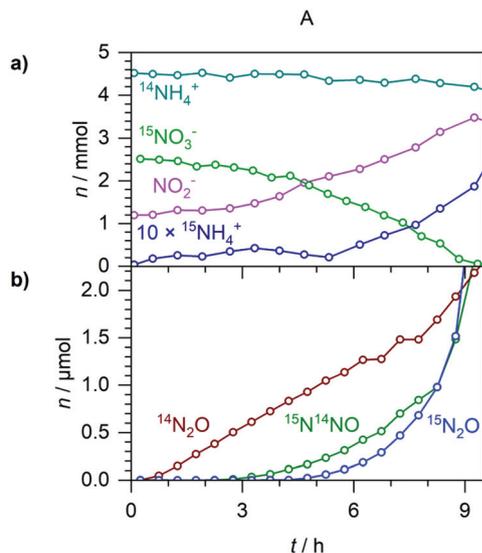
$\text{NO}_2^-$ . The most notable difference between 10 mM  $^{15}\text{NO}_3^-$  reduction (discussed in section 3.2), and 5 mM  $^{15}\text{NO}_3^-$  reduction was  $\text{H}_2$  production that occurred after  $\text{NO}_2^-$  depletion in Fig. 7. No  $\text{H}_2$  production was observed during A' as formate-dependent  $\text{NO}_2^-$  reduction likely made the intracellular formate unavailable for FHL induction. The presence of formate is required for FHL expression but it can be made unavailable by coupling to the reduction of electron acceptors. This inhibiting effect has been observed for  $\text{NO}_3^-$  and trimethylamine *N*-oxide respiring *E. coli* cultures and in both cases the effect could be partially relieved by adding exogenous formate.<sup>56,58</sup> When  $\text{NO}_2^-$  was depleted, 5.2 mmol glucose remained meaning further formate could be produced during B' which may have triggered the induction of FHL. From 9–15 h, 6.0 mmol  $\text{H}_2$  and a further 6.0 mmol  $\text{CO}_2$  were produced from the disproportionation of formate. At 10 h, there was a peak of 2.1 mmol formate excreted. During B', a further 5.3 mmol acetate and 3.5 mmol ethanol were produced. By 12 h, the pH dropped to 5.4 and then remained stable as 1.6 mmol acetate was produced and balanced by the reimport and disproportionation of 1.5 mmol formate. By 14 h, the  $\text{OD}_{600}$  peaked at 1.5, just before the end of bacterial activity at 15 h due to the depletion of glucose and formate. 42.8 out of the 45 mmol C from glucose can be accounted for in the biomass ( $\sim 8$  mmol C), 8.9 mmol  $\text{CO}_2$ , 9 mmol acetate (18 mmol C) and 3.8 mmol ethanol (7.6 mmol C). During exponential growth,  $^{14}\text{NH}_4^+$  decreased from 4.5 to 2.9 mmol as did  $^{15}\text{NH}_4^+$  from a peak value of 1.15 to 0.9 mmol accounting for 1.85 mmol out of the  $\sim 2$  mmol N in the biomass.

### 3.4 Simultaneous nitrate and nitrite reduction

In section 3.2, when *E. coli* was supplemented with  $\text{NO}_3^-$ , there was a distinct hierarchy of metabolic pathways between phases A, B and C.  $\text{NO}_3^-$  reduction dominated in A, followed by  $\text{NO}_2^-$  reimport and reduction in B and finally fermentation in C. However, in A it was observed that some  $\text{NO}_2^-$  was simultaneously reduced alongside  $\text{NO}_3^-$  to  $\text{NH}_4^+$  and  $\text{N}_2\text{O}$ . To further investigate the overlap between the reductions of  $\text{NO}_3^-$  and  $\text{NO}_2^-$  in A, anaerobic *E. coli* was supplemented with 10 mM  $^{15}\text{NO}_3^-$  and 5 mM  $^{14}\text{NO}_2^-$  as shown in Fig. 8 and 9. Phase A (0–9 h) lasted until all  $^{15}\text{NO}_3^-$  was reduced to  $^{15}\text{NO}_2^-$ . Phase B (9–30 h) corresponded to the reduction of  $\text{NO}_2^-$  to  $\text{NH}_4^+$  with concurrent  $\text{N}_2\text{O}$  production *via* NO. At 15.5 h, the  $\text{OD}_{600}$  peaked and exponential growth of *E. coli* ended; thus, phase B1 (9–15.5 h) was  $\text{NO}_2^-$  reduction with glucose still present and phase B2 (15.5–30 h) was  $\text{NO}_2^-$  reduction during glucose depletion. Fig. 8 displays  $n$  of  $^{15}\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $^{14}\text{NH}_4^+$ ,  $^{15}\text{NH}_4^+$  ( $\times 10$ ) and  $\text{N}_2\text{O}$  isotopomers in A. The complete characterization of bacterial growth is given in Fig. 9.

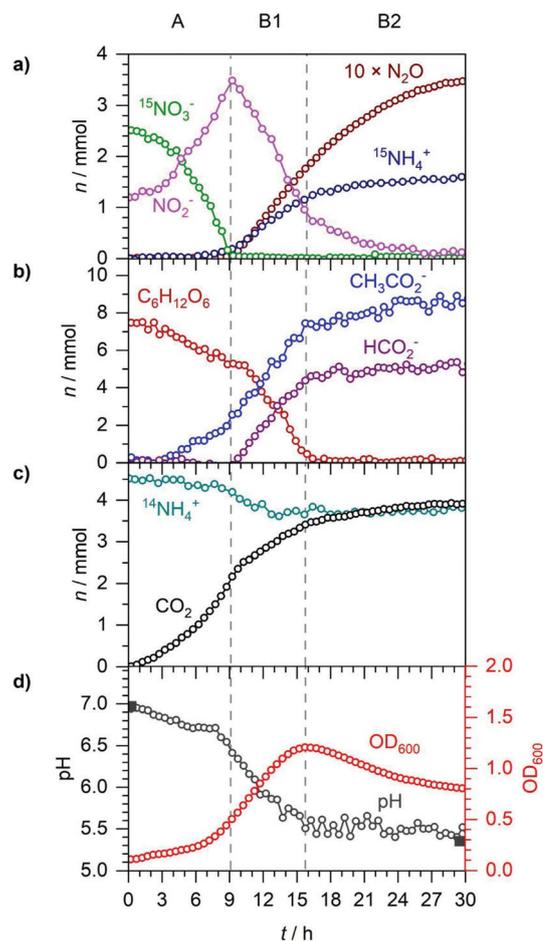
In phase A, 2.5 mmol  $^{15}\text{NO}_3^-$  was reduced and *ca.* 2.25 mmol  $^{15}\text{NO}_2^-$  was excreted.  $\text{NO}_2^-$  colorimetry cannot distinguish between  $^{14}\text{NO}_2^-$  and  $^{15}\text{NO}_2^-$ , so  $\text{NO}_2^-$  was observed to increase from 1.25 to 3.5 mmol. During A, as in section 3.2, some  $\text{NO}_2^-$  was reduced alongside  $^{15}\text{NO}_3^-$  to 0.2 mmol  $^{15}\text{NH}_4^+$  and  $\text{N}_2\text{O}$  isotopomers.  $^{14}\text{NO}_2^-$  reduction to  $^{14}\text{N}_2\text{O}$  occurred immediately, with 2.2  $\mu\text{mol}$   $^{14}\text{N}_2\text{O}$  produced almost linearly by





**Fig. 8**  $\text{N}_2\text{O}$  isotopomers produced in the  $\text{NO}_3^-$  reduction phase (A) of anaerobic *E. coli* growth in M9 medium supplemented with 10 mM  $^{15}\text{NO}_3^-$  and 5 mM  $^{14}\text{NO}_2^-$ . (a) Time-dependent number of moles ( $n$ ) of  $^{15}\text{NO}_3^-$ ,  $\text{NO}_2^-$ ,  $^{14}\text{NH}_4^+$  and  $^{15}\text{NH}_4^+$  ( $\times 10$ ). (b)  $n$  of  $\text{N}_2\text{O}$  isotopomers produced.  $^{14}\text{N}^{15}\text{NO}$  is omitted due to essentially having the same behaviour as  $^{15}\text{N}^{14}\text{NO}$ .

9 h. This indicated that even before  $\text{NO}_3^-$  reduction began, some unknown enzymatic activity to reduce small quantities of  $\text{NO}_2^-$  to  $\text{N}_2\text{O}$  was immediately active. For the first 3 h,  $^{15}\text{NO}_3^-$  and  $\text{NO}_2^-$  measurements were virtually constant suggesting a lag in the expression of NRA. This lag was best indicated by the highly sensitive positional isomers  $^{14}\text{N}^{15}\text{NO}$  and  $^{15}\text{N}^{14}\text{NO}$  which were not detected until  $^{15}\text{NO}_2^-$  was made available by  $^{15}\text{NO}_3^-$  reduction starting from 3 h.  $^{15}\text{N}_2\text{O}$  production also began at 3 h, but much slower than the production of  $^{14}\text{N}_2\text{O}$  and the positional isomers, due to  $^{14}\text{NO}_2^-$  initially being more readily available than  $^{15}\text{NO}_2^-$ . By the end of A, 1.5  $\mu\text{mol}$  each of  $^{14}\text{N}^{15}\text{NO}$ ,  $^{15}\text{N}^{14}\text{NO}$  and  $^{15}\text{N}_2\text{O}$  were produced alongside the 2.2  $\mu\text{mol}$   $^{14}\text{N}_2\text{O}$ , totalling 6.7  $\mu\text{mol}$ . It is unknown if  $^{14}\text{NO}_2^-$  was also immediately reduced to  $^{14}\text{NH}_4^+$ , due to the large background of 4.5 mmol  $^{14}\text{NH}_4^+$  in the growth medium. It can be assumed *ca.* 0.25 mmol  $\text{NO}_2^-$  was reduced during A based on the  $\text{NO}_2^-$  colorimetry measurements giving a partitioning of 5%  $\text{NO}_2^-$  reduced to  $\text{N}_2\text{O}$ , instead of  $\text{NH}_4^+$ . This was a higher value than the 1% observed during A in section 3.2, indicating that the added  $^{14}\text{NO}_2^-$  led to more NO generation and detoxification to  $\text{N}_2\text{O}$ . During A, glucose decreased from 7.5 to 5.6 mmol due to the production of 2.1 mmol  $\text{CO}_2$ , 2.0 mmol acetate and biomass synthesis. The  $\text{OD}_{600}$  began increasing indicating exponential bacterial growth while acetate excretion caused the pH to decrease from 7.0 to 6.5. No formate was excreted during A, as the  $n$  of  $\text{CO}_2$  and acetate suggested all formate formed was oxidised to  $\text{CO}_2$ . No ethanol was detected during the entire 30 h experiment, likely due to the abundance of electron acceptors to couple reducing equivalents to.



**Fig. 9** Anaerobic *E. coli* growth in M9 medium supplemented with 10 mM  $^{15}\text{NO}_3^-$  and 5 mM  $^{14}\text{NO}_2^-$ . A to C denotes three distinct phases:  $\text{NO}_3^-$  reduction (A),  $\text{NO}_2^-$  reduction with glucose present (B1) and  $\text{NO}_2^-$  reduction with glucose depleted (B2). (a) Time-dependent number of moles ( $n$ ) of  $^{15}\text{NO}_3^-$ ,  $\text{NO}_2^-$  (both  $^{14}\text{N}$  and  $^{15}\text{N}$ ),  $^{15}\text{NH}_4^+$  and sum of all  $^{14}\text{N}/^{15}\text{N}$ -isotopomers of  $\text{N}_2\text{O}$  ( $\times 10$ ). (b)  $n$  of glucose, acetate and formate. (c)  $n$  of  $\text{CO}_2$  and  $^{14}\text{NH}_4^+$ . (d) Spectroscopically determined pH (open circles), externally measured pH (solid squares) and  $\text{OD}_{600}$ .

Phase B1 began with  $^{15}\text{NO}_3^-$  depletion and ended at 15.5 h when glucose was depleted, coinciding with the  $\text{OD}_{600}$  peaking at 1.2. The pH dropped further to 5.6 due to the excretion of 5.0 mmol formate and a further 6.0 mmol acetate. The sum of formate excreted and the further 1.3 mmol  $\text{CO}_2$  produced was in good agreement with the amount of acetate excreted.

Phase B2 lasted until  $\text{NO}_2^-$  depletion at 30 h. From  $\text{NO}_2^-$  reduction, 1.6 mmol  $^{15}\text{NH}_4^+$  and 0.35 mmol  $\text{N}_2\text{O}$  were produced overall. The final composition of  $\text{N}_2\text{O}$  isotopomers was previously introduced in Fig. 5c. As the majority of  $\text{N}_2\text{O}$  production occurred in B when the  $\text{NO}_2^-$  composition was *ca.* 66%  $^{15}\text{NO}_2^-$  and 33%  $^{14}\text{NO}_2^-$ , a near statistical mixture of  $\text{N}_2\text{O}$  isotopomers was formed of 0.17 mmol  $^{15}\text{N}_2\text{O}$  (49%), 0.08 mmol  $^{15}\text{N}^{14}\text{NO}$  (22%), 0.07 mmol  $^{14}\text{N}^{15}\text{NO}$  (20%) and 0.03 mmol  $^{14}\text{N}_2\text{O}$  (9%). For comparison, a perfect statistical mixture would have produced 44.4%  $^{15}\text{N}_2\text{O}$ , 22.2%  $^{15}\text{N}^{14}\text{NO}$ , 22.2%  $^{14}\text{N}^{15}\text{NO}$  and 11.1%  $^{14}\text{N}_2\text{O}$ . It is unknown whether the



slight preference for  $^{15}\text{N}^{14}\text{NO}$  over  $^{14}\text{N}^{15}\text{NO}$  is significant or due to experimental uncertainty. The partitioning of the 3.5 mmol  $\text{NO}_2^-$  to  $\text{N}_2\text{O}$  in **B** was 20%, a much higher value than the 10% observed during **A** in section 3.2. This is consistent with previous studies that found that between 5–36% of  $\text{NO}_3^-$  is converted to  $\text{N}_2\text{O}$  by *E. coli*, depending on growth conditions.<sup>35</sup> During **B2**,  $\text{CO}_2$  increased by a further 0.5 mmol while the pH remained constant at 5.6 due to no significant change in acetate and formate. 33 out of the 45 mmol C from glucose can be accounted for in the biomass (~8 mmol C), 4 mmol  $\text{CO}_2$ , 8 mmol acetate (16 mmol C) and 5 mmol formate. The higher  $\text{NO}_2^-$  content may have had cytotoxic effects in *E. coli* resulting in other products that have not been accounted for in the C balance. During **B2**, the  $\text{OD}_{600}$  dropped from 1.2 to 0.8 suggesting cell death or changes in cellular size and morphology, possibly due to the cytotoxicity of  $\text{NO}_2^-$  and NO. The 2.5 mmol  $^{15}\text{N}$  label was accounted for in the 1.6 mmol  $^{15}\text{NH}_4^+$ , 0.17 mmol  $^{15}\text{N}_2\text{O}$  (0.34 mmol  $^{15}\text{N}$ ), 0.08 mmol  $^{15}\text{N}^{14}\text{NO}$ , 0.07 mmol  $^{14}\text{N}^{15}\text{NO}$  and ca. 0.5 mmol  $^{15}\text{NH}_4^+$  assumed to have been used for biosynthesis. As ca. 2.0 mmol  $\text{NH}_4^+$  was needed for biosynthesis, it was assumed ca. 1.5 mmol was taken from  $^{14}\text{NH}_4^+$ , which decreased overall from 4.5 to 4.0 mmol suggesting ca. 1.0 mmol  $^{14}\text{NH}_4^+$  produced from the reduction of the 1.25 mmol  $^{14}\text{NO}_2^-$ . This was in good agreement with the 0.26 mmol  $^{14}\text{NO}_2^-$  reduced to  $\text{N}_2\text{O}$  isotopomers with 0.03 mmol  $^{14}\text{N}_2\text{O}$  (0.06 mmol  $^{15}\text{N}$ ), 0.08 mmol  $^{15}\text{N}^{14}\text{NO}$  and 0.07 mmol  $^{14}\text{N}^{15}\text{NO}$ .

## 4. Conclusions

We have studied  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reduction during DNRA by anaerobic *E. coli* batch cultures by a combination of advanced spectroscopic analytical techniques in conjunction with  $^{15}\text{N}$ -isotopic labelling. The online spectroscopic techniques described here are non-invasive, avoiding any contact with the bacterial suspension, and provide concentrations in real-time. We discussed in detail the spectroscopy, which spectral features are most useful for analysis, and data analysis and fitting routines for quantitative analysis. *In situ* analysis of the headspace is achieved using cavity-enhanced Raman (CERS) and long-path White cell FTIR spectroscopies alongside liquid-phase Raman spectroscopy. Gas phase CERS allows  $\text{CO}_2$ ,  $\text{H}_2$ ,  $\text{N}_2$  and  $\text{O}_2$  monitoring while White cell FTIR measures  $\text{CO}_2$ , ethanol and  $\text{N}_2\text{O}$ . The 6 m pathlength White cell affords trace gas detection of  $\text{N}_2\text{O}$  with a noise equivalent detection limit of 60 nbar or 60 ppbv in 1 atm ( $1\sigma$  noise equivalent, 128 scans corresponding to 120 s acquisition). This extremely high sensitivity could be utilised in situations where  $\text{N}_2\text{O}$  cannot be allowed to build up, e.g. in continuous culture studies. Quantitative analysis is discussed for all four  $^{14}\text{N}/^{15}\text{N}$ -isotopomers, including the positional isomers  $^{14}\text{N}^{15}\text{NO}$  and  $^{15}\text{N}^{14}\text{NO}$ , a unique capability not available to other analytical techniques.

$^{15}\text{N}$ -isotopic labelling of  $\text{NO}_3^-$  identifies the sources of N-atoms in products of *E. coli* metabolism, in particular, it pro-

vides insight into the mechanism of  $\text{N}_2\text{O}$  production during mixed  $\text{NO}_3^-$  and  $\text{NO}_2^-$  reduction. This study is one of very few reporting quantitative analysis of  $\text{N}_2\text{O}$  production by *E. coli* under various conditions. The reductions of  $^{15}\text{NO}_3^-$ ,  $^{15}\text{NO}_2^-$ , and mixed  $^{15}\text{NO}_3^-$  and  $^{14}\text{NO}_2^-$  to  $\text{NH}_4^+$  and  $\text{N}_2\text{O}$  have been discussed. In a major pathway,  $\text{NO}_3^-$  is reduced to  $\text{NH}_4^+$  via  $\text{NO}_2^-$ , with the bulk of  $\text{NO}_2^-$  reduction occurring after  $\text{NO}_3^-$  depletion. By isotopically labelling  $^{15}\text{NO}_3^-$ ,  $^{15}\text{NH}_4^+$  production is distinguished from background  $^{14}\text{NH}_4^+$  in the growth medium. In a minor pathway,  $\text{NO}_2^-$  is reduced to  $\text{N}_2\text{O}$  via the toxic radical NO. With excellent detection sensitivities,  $\text{N}_2\text{O}$  monitors trace  $\text{NO}_2^-$  reduction even when cells are predominantly reducing  $\text{NO}_3^-$ ; the analysis of  $\text{N}_2\text{O}$  isotopomers reveals that some enzymatic  $\text{NO}_2^-$  reduction activity occurs immediately for cultures supplemented with mixed  $^{15}\text{NO}_3^-$  and  $^{14}\text{NO}_2^-$ . Optical density and pH measurements are discussed in context of acetate, formate and  $\text{CO}_2$  production.  $\text{H}_2$  production is repressed by  $\text{NO}_3^-$ , but with  $\text{NO}_2^-$  only, CERS detects  $\text{H}_2$  produced by formate hydrogenlyase after  $\text{NO}_2^-$  depletion.

In future work, we want to extend our spectroscopic approach to monitor different bacterial pathways, in particular, the relationship between fermentative and other respiratory pathways and to study nitrifying and denitrifying bacteria. These spectroscopic techniques are capable of detecting key species in the nitrogen cycle and with the ability to sensitively distinguish  $\text{N}_2\text{O}$  isotopomers they may be of great interest for helping better understand global  $\text{N}_2\text{O}$  budgets. Spectroscopic monitoring of bioprocesses has excellent potential to supplement or replace traditional techniques in analytical chemistry.

## Author contributions

All authors have contributed equally to the conception of the project, the experimental work, the analysis and to the writing of the manuscript.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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