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COMMUNICATION

## Facile and fast detection of bacteria via the detection of exogenous volatile metabolites released by enzymatic hydrolysis†

Laure-Hélène Guillemot,<sup>a,b</sup> Marjorie Vrignaud,<sup>a</sup> Pierre R. Marcoux,<sup>\*a</sup> Charles Rivron<sup>b</sup> and Thu-Hoa Tran-Thi<sup>\*b</sup>

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A low-cost, innovative and non-invasive colorimetric test, which can be universally used, is proposed to detect pathogen bacteria via the simple and fast detection of volatile metabolites released by enzymatic hydrolysis. The proof of concept is shown via three sets of experiments studying the release of the p-nitrophenol metabolite in solution in the *E. coli* cultures containing 4-nitrophenyl- $\beta$ -D-glucuronide, the trapping efficiency of the gaseous metabolite by various tailored and functionalized xerogels, and the trapping and detection of gaseous p-nitrophenol released by *E. coli* bacteria.

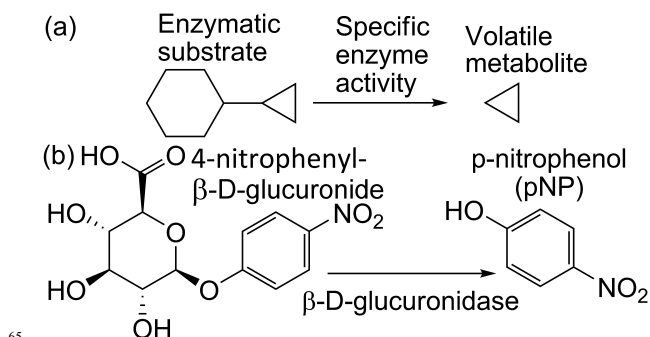
### Introduction

Bacterial contamination of food with pathogens is a major public health concern and a financial burden in food industries. In the summer of 2008, the presence of *Listeria monocytogenes* in ready-to-eat meat caused a listeriosis outbreak, which resulted in the deaths of 23 Canadians. The recall of the contaminated meats had cost 20 million euros to the manufacturer.<sup>1</sup> Following this tragic event, the Government of Canada asked Sheila Weatherill to lead an independent investigation into the circumstances of the outbreak and to make recommendations to strengthen the food safety system. Among the actions that the Canadian government has taken to implement all the recommendations of the Weatherill report are investments of \$100 million over five years in inspector training, tools and technology, and science capacity.<sup>2</sup> The more recent 2011 outbreak of Shiga-toxin *E. coli* O104:H4 in Europe with the ready-to-eat sprouts, which wrongly involved the Spanish cucumbers, confirms again the need of reliable and easy-to-use methods for early stage detection of pathogens.

The challenge for the future is to detect the food contamination in the very early stages of the food processing. Currently, the food samples are sent to a microbiology laboratory for costly and time-consuming analysis (6 to 7 days), during which the food could have already reached the consumer. In response to this need, many research laboratories had proposed new analytical methods. Henry's group proposed a paper-based analytical device for colorimetric detection of *E. coli* O157:H7, *Salmonella typhimurium* and *L. monocytogenes*.<sup>3</sup> The paper-based microspots are produced by wax-printing on filter paper and the detection is achieved by measuring the color change when an enzyme associated with the pathogen of interest, reacts with a

chromogenic substrate. The authors can obtain, with 12 hours of enrichment procedures, a positive test with samples inoculated with 10<sup>1</sup> colony forming units/cm<sup>2</sup>. Though this method is interesting in terms of simplicity, rapidity and cost, there is still one important drawback, regarding the application to colored food samples; the presence of blood, wine, tomato sauce or food colorants could compromise the colorimetric test.

To overcome this drawback, Snyder *et al.*<sup>4</sup> suggested the use of enzymatic substrates carrying a volatile moiety. Their hydrolysis by a specific enzyme, produced by pathogens, leads to the formation of a volatile metabolite, which is then detected by gas chromatography coupled with ion mass spectrometry (GC-IMS). A gas chromatography-mass spectrometry method (GC-MS) that reaches nanomolar detection limits was also reported,<sup>5</sup> but it involves a liquid-liquid extraction. Furthermore, GC-IMS and GC-MS are costly techniques which are time-consuming and need high technical assistance.



**Fig. 1** (a) Enzymatic hydrolysis of enzymatic substrate releasing volatile metabolite. (b) Hydrolysis of 4-nitrophenyl- $\beta$ -D-glucuronide (pNPG) releasing colored volatile p-nitrophenol.

Detecting a targeted analyte in liquid samples can be also achieved at nanomolar level with electrochemistry, when exploiting the large adsorption surfaces of nanostructured microelectrodes.<sup>6</sup> However, this method requires the oxidation or reduction potentials of the targeted analyte to be lower than those of other oxidizable or reducible compounds released by bacteria.

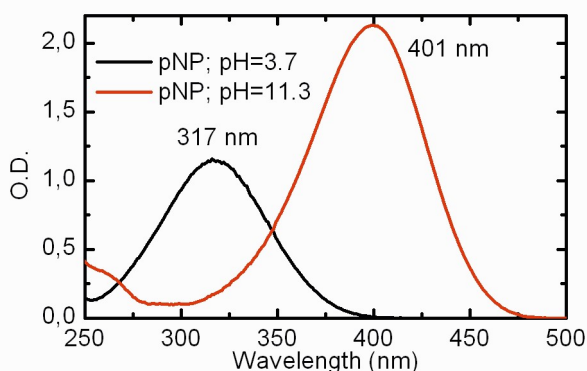
In this work, we propose to replace GC-IMS and GC-MS by a simple and innovative method of detection of exogenous volatile metabolites released by enzymatic hydrolysis (Figure 1a). It is based on the use of transparent porous matrices, whose functions are threefold: i) the pore size is tailored to trap the volatile

metabolite and the matrix acts as a sponge to concentrate the analyte, <sup>7</sup> ii) the pore cavities are engineered to chemically change the metabolite and improve the detection and iii) the transparent matrix allows a quantitative measurement of the metabolites with optical absorption measurements.

Monolithic xerogels were used in the past to trap and detect gaseous compounds such as formaldehyde and aromatic monocyclic hydrocarbons, <sup>8</sup> which are mainly air pollutants for the control of air quality. For such purpose, the sensors are often requested to work over a humidity range of 20 to 60%. The detection of volatile compounds released from culture media implies the use of sensors which could detect volatile compounds in very humid environments, with a relative humidity of 100%. The proof of concept is here given with the study of the release of p-nitrophenol (pNP) by hydrolysis of 4-nitrophenyl- $\beta$ -D-glucuronide by  $\beta$ -glucuronidase (Figure 1b), an enzyme associated with *E. coli*. <sup>9</sup> The present proof of concept includes four studies: i) the release of pNP from 4-nitrophenyl- $\beta$ -D-glucuronide in solution, ii) the production of porous matrices tailored to trap the analyte, iii) the trapping of gaseous pNP and the analysis of the local intrapore environment, iv) the trapping of released pNP above various bacteria cultures.

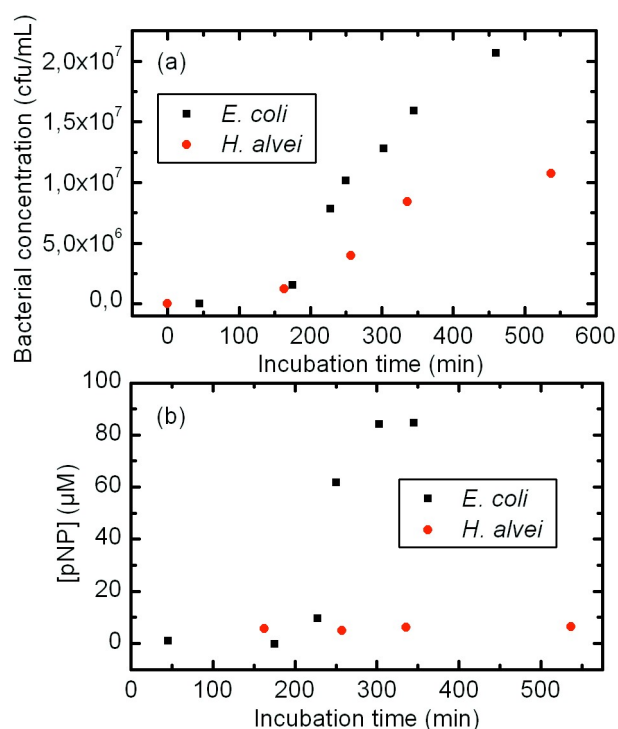
## Results and discussion

Para-nitrophenol (pNP) was chosen as volatile metabolite for 4 reasons. i) The value of dimensionless Henry's constant ( $H_{cc}=4,13 \cdot 10^{-5}$ ) is high enough, <sup>10</sup> implicating a non-negligible concentration of gaseous pNP in the headspace above the aqueous bacterial culture. ii) pNP displays interesting optical properties. As shown in Figure 2, the acidic form of pNP is colorless ( $\lambda_{max}=317$  nm,  $\epsilon_{317nm}=8300$  L.mol<sup>-1</sup>.cm<sup>-1</sup>), while the anionic form absorbs in the visible region, with a yellow color ( $\lambda_{max}=401$  nm,  $\epsilon_{401nm}=18000$  L.mol<sup>-1</sup>.cm<sup>-1</sup>). iii) Bacteria do not naturally emit pNP and therefore, there will be no chemical "background/interference". iv) The substrate that releases pNP (4-nitrophenyl- $\beta$ -D-glucuronide, pNPG) and pNP are commercially available. Furthermore, numerous other substrates carrying pNP and targeting other enzymatic activities are also available. Therefore, many pathogen bacteria can be detected with this volatile metabolite. For example, we can target  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\beta$ -alanyl-arylamidase and C8-esterase for *E. coli*, *S. aureus*, *P. aeruginosa*, and *Salmonella* detection, respectively. <sup>9</sup>



**Fig. 2** Absorption spectra of pNP under its anionic and acidic forms in aqueous solutions.  $pK_a$  (pNP)=7.15.

A preliminary study of the release of pNP by bacteria strains able (or not) to produce  $\beta$ -D-glucuronidase enzyme was carried out in liquid nutrient media. 2-(N-morpholino)ethanesulfonic acid (MES) buffered culture media containing pNPG were inoculated with  $10^5$  cfu/mL of different bacteria strains and incubated at 37°C with 250 rpm stirring (refer to electronic supplementary information for culture media descriptions). At different incubation times, a culture sample was pulled out and its absorption spectrum was acquired to follow the bacterial growth. Indeed, a previous study has shown that a value of 0.1 for the optical density at 550 nm corresponds to  $2 \times 10^7$  cfu/mL. <sup>11</sup> The sample was then filtered to discard bacterial cells and NaOH was added in order to obtain the released pNP uniquely in its anionic form. The absorption spectrum of the so-obtained sample was collected and the concentration of released pNP calculated from the absorbance value at  $\lambda_{max}=401$  nm.



**Fig. 3** (a) Bacterial growth with time for *Escherichia coli* ATCC 11775 and *Hafnia alvei* ATCC13337. (b) Concentration of free pNP in MES culture medium for different incubation times.

**Table 1** Maximum pNP concentration for  $\beta$ -D-glucuronidase ( $\beta$ -GUR) positive and negative species.

	$\beta$ -GUR activity <sup>a</sup>	generation time (min)	[pNP]max ( $\mu$ M)
<i>E. coli</i> ATCC 11775	positive	24	85
<i>E. coli</i> ATCC 35421	positive	17	70
<i>H. alvei</i> ATCC 13337	negative	34	6
<i>E. cloacae</i> ATCC 13047	negative	28	6
<i>C. freundii</i> ATCC 8090	negative	31	6

<sup>a</sup>  $\beta$ -GUR activities were tested using the API 20E system (bioMérieux).

As shown in Figure 3(a), pNPG substrate and released pNP do

not affect the bacterial growth since both  $\beta$ -D-glucuronidase positive strain such as *E. coli* and  $\beta$ -D-glucuronidase negative strain such as *H. alvei* display typical times of generation. As  $\beta$ -D-glucuronidase activity is specific to *E. coli*, the free pNP concentration increases with incubation time in *E. coli* culture medium (Figure 3(b)). As expected, pNP is not released in the culture medium inoculated with *H. alvei* (Figure 3(b)). Table 1 summarizes the results obtained for other bacteria strains. It can be seen that the concentration of released pNP is high, from 70 to 85  $\mu$ M for both *E. coli* strains. The value of 6  $\mu$ M of pNP determined for all  $\beta$ -D-glucuronidase negative strains corresponds to an artefact due to the small contribution, at 401 nm, of the tail of the wide absorption band of the pNPG substrate.

In order to trap and detect the volatile portion of pNP released by *E. coli* strains, transparent xerogels are synthesized using the sol-gel process. The process consists in the hydrolysis of silicon precursors such as tetramethylorthosilicate (TMOS) which condense into a three-dimensional network, leading to a nanoporous material. Functionalized silicon precursors such as (3-aminopropyl)triethoxysilane (APTES) or 3-methoxypropyltrimethoxysilane (PEG-1) can be used to modify the intrinsic pH of the pores and to tailor the diameter of the cavities.

In this work, 3% of PEG-1, 3% or 6% of APTES were mixed to TMOS with methanol and deionized water according the following molar ratio: Si precursors/MeOH/H<sub>2</sub>O: 1/5/4 (refer to electronic supplementary information for synthesis protocols). These three formulations lead to the production of porous xerogels which are transparent over the 300-800 nm domain. Their porosity properties (Table 2) were determined by establishing the isotherms of adsorption and desorption of nitrogen at 77 K and using both BET (Brunauer, Emmett and Taylor) and DFT analytical methods. The xerogels display high specific adsorption surface areas which provide the "sponge" property, necessary to concentrate the targeted analyte. It is noteworthy that xerogels doped with PEG-1 exhibit a smaller pore size ( $\sim$ 10 Å) and a lower specific adsorption surface area (466 m<sup>2</sup>/g) than xerogels doped with APTES, due to the hindrance effect of the polymer as compared to the amine chain. On the other hand, increasing the amount of APTES, which displays a functional group, unable to condense with the other silanol groups to form the tridimensional network, results in a pore size decrease.

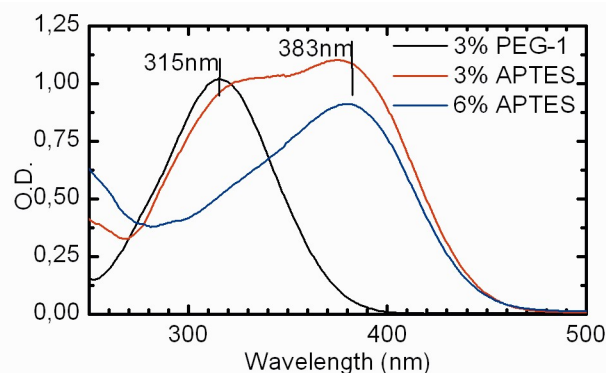
**Table 2** Porosity properties of the xerogel monoliths obtained from the isotherm of adsorption of N<sub>2</sub> at liquid temperature.

	APTES 3%	APTES 6%	PEG-1 3%
Specific adsorption surface area (m <sup>2</sup> /g)	1 067	1 003	466
Pore volume (mL/g) <sup>a</sup>	1.00	0.76	0.26
Pore diameter distribution (Å) <sup>a</sup> :	34 – 58 Å	27 – 57 Å	10 – 32 Å
range / maximum	max: 49 Å	max: 33 Å and 47 Å	max: 10 Å

<sup>a</sup> using B.E.T and DFT analytical methods.

The influence of the local intrapore environment on the pNP properties is studied, by exposing the xerogels to 100 ppm of pNP chemical vapors. The pNP atmosphere was produced in a closed system where a drop of pNP in ethanolic solution was deposited

on a warm metallic surface (refer to electronic supplementary information for protocol descriptions). Both ethanol and pNP are evaporated and trapped in the xerogels. Figure 4 displays the absorption spectra of pNP trapped in xerogels doped with 3% APTES, 6% APTES and 3% PEG-1, respectively, after 24 hour exposure to 100 ppm of pNP. In the matrix doped with PEG, which provides an aprotic environment, the pNP absorption band corresponds to the acidic form of pNP with a maximum shifted to the blue ( $\lambda_{\text{max}}=315$  nm,  $\Delta\nu=200$  cm<sup>-1</sup>), as compared with pNP in acidic aqueous solution ( $\lambda_{\text{max}}=317$  nm). With APTES precursor, which provides an "alkaline" environment with the propylamine chain, the absorption spectrum of pNP displays a band peaking at  $\lambda=383$  nm and two large bands centered at  $\lambda=321$  nm and  $\lambda=383$  nm, for 6% and 3% of APTES, respectively. The absorption band peaking at 383 nm corresponds to pNP anionic form and is blue-shifted as compared to the one in aqueous solution ( $\Delta\nu=1172$  cm<sup>-1</sup>). With 3% APTES, the pNP and pNP<sup>-</sup> forms coexist, but in a minor proportion for the acidic form.



**Fig. 4** Absorption spectra of various monoliths exposed to 100 ppm of gaseous pNP.

When the matrices are exposed to pNP in humid conditions, we noted that the trapping of pNP is slower due to the competitive diffusion of water vapor and gaseous pNP into the monolith. Therefore, xerogels doped with a high proportion of APTES (6%) will not be used for further studies since the alkaline property enhances the hydrophilic character of the matrix and thus favors the trapping of water. As PEG-1 doped xerogels adsorbs pNP under its colorless form, we will favor the use of 3% APTES doped xerogels in the coming experiments. This first result shows how important the choice of functionalized silicon precursors is for a good trapping and optical detection of pNP.

Our next objective is to demonstrate the possibility of trapping and optically detect, in the headspace over a bacterial culture, volatile pNP issued from the specific enzymatic activity of bacteria. The bacteria cultures, containing pNPG buffered with either 3-(N-morpholino)propanesulfonic acid (MOPS; pH=7.33) or 2-(N-morpholino)ethanesulfonic acid (MES; pH=6.1), were inoculated with *E. coli* ATCC 11775 (respectively 2.10<sup>5</sup> cfu/mL and 10<sup>5</sup> cfu/mL; refer to electronic supplementary information for protocols). A 3% APTES doped xerogel was placed above these cultures as shown in Figure 5(a).

After 16 hours of exposure at 37°C, the xerogel placed above a MOPS buffered *E. coli* culture has turned yellow which indicates that pNP has been trapped (Figure 5b). The shape of the absorption spectrum witnesses the presence of pNP trapped in its

anionic form. From the absorbance at 383 nm and 330 nm, the [pNP<sup>-</sup>] and [pNP] concentrations can be deduced (17 μM and 22 μM respectively), which corresponds to a total of 4.9 nmol for both acid and basic states of pNP trapped in the monolith (volume = 125 μL). After 16 hours of incubation, the expected concentration of pNP in the culture is 85 μM as shown earlier. According to Henry's law, and taking the value of  $H_{CC}=4,13 \cdot 10^{-5}$ , the amount of pNP at equilibrium in the gas phase above the solution is only 0.022 nmol. The amount of the trapped pNP being 220 times higher than the expected one is explained in terms of a displacement of the gas-liquid equilibrium due to the xerogel acting as a sponge.

As a control experiment to check the potential interference of others volatile compounds emitted by the bacteria, we expose a xerogel to a culture medium, non-supplemented with pNPG enzymatic substrate (refer to electronic supplementary information). The absorption spectrum of the exposed xerogel displays in the UV domain a new and narrow absorption band, peaking at around 280 nm. This metabolite does not interfere with pNP since there is a large gap of 100 nm between both absorption bands. We could identify this metabolite as indole by performing three experiments. The xerogel was first ground and the trapped compound is extracted with methanol; the absorption spectrum of the alcoholic solution corresponds to that of indole in solution. To strengthen this hypothesis, the specific reaction of 4-(dimethylamino)-cinnamaldehyde, DMACA, with indole was applied, <sup>7</sup> by adding DMACA to the solution. The distinctive blue-green color observed can be attributed to the azafulvenium chloride salt, formed from the reaction of indole with DMACA, thus confirming our hypothesis. In the last experiment, the xerogel was exposed to cultures of bacteria (*H. alvei*, *P. putida*) that do not produce indole and no absorption band around 280 nm was detected in that case.

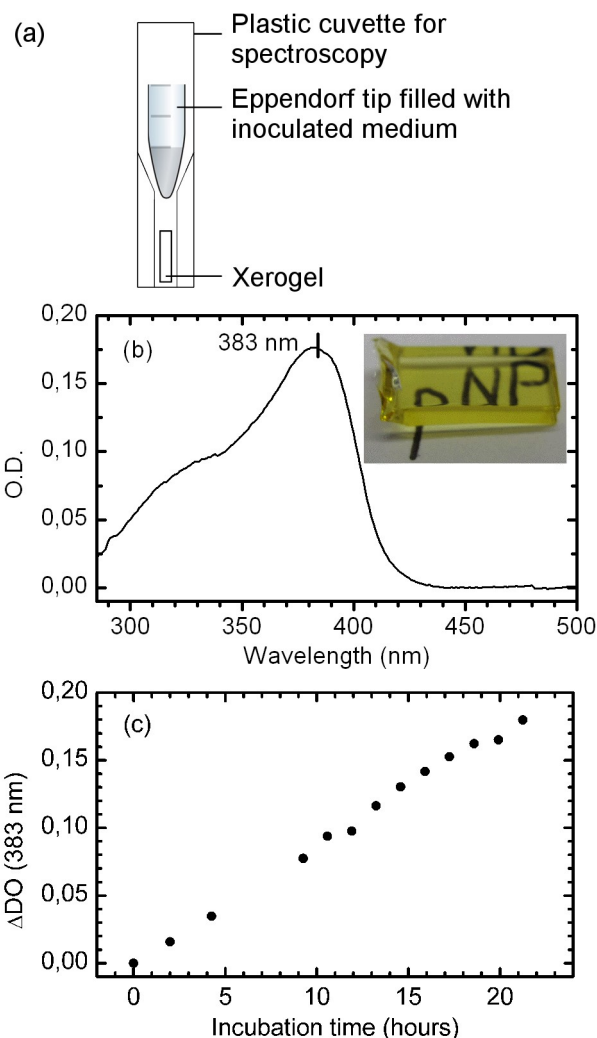
A xerogel is also exposed at 37°C to a MES buffered *E. coli* culture. This buffer is preferred to MOPS as MES ( $pK_a=6.1$ ) favors the molecular form of pNP ( $pK_a=7.15$ ) in aqueous solution, which is volatile. The pNP trapping is monitored by following at 383 nm, as a function of time, the absorption increase of pNP<sup>-</sup>. As shown in Figure 5(c), this absorbance grows linearly with the incubation time. It can be seen that 1 hour of exposure is sufficient to detect pNP<sup>-</sup> since the detection limit is  $\Delta OD \sim 0.01$ .

When comparing the efficiency of our method with Snyder's results and starting with the same initial concentration of  $10^5$  cfu/mL, it can be noted that the GC-IMS technique allows a faster detection of o-nitrophenol within 15 minutes. <sup>4</sup> The faster response obtained by Snyder's group might be due to the high volatility of o-nitrophenol, the Henry's constant being 14 times higher for o-nitrophenol ( $5.85 \cdot 10^{-4}$ ) than for pNP ( $4.13 \cdot 10^{-5}$ ). <sup>10</sup> We therefore bring an improvement since the time-to-detection is not significantly slower than GC-IMS, using a much lighter and low-cost instrumentation and a less volatile analyte. New experiments are foreseen in the future, with a substrate carrying o-nitrophenol, to check the potential improvement of the sensor sensitivity. In comparison with other techniques of bacteria detection performed in gas-phase such as radiometric respirometry, starting with the same initial concentration of  $10^5$  cfu/mL, our technique is faster than the 1 hour limit of detection

of <sup>14</sup>CO<sub>2</sub> level. <sup>12</sup> In addition, we do not generate radioactive waste.

## Conclusion

With the sets of experiments aimed at proving a new concept, we demonstrated the possibility of detecting, with a simple, non invasive and low cost method, bacteria species by using an enzymatic substrate and functionalized xerogels. This concept can be universally applied; it only requires the identification of the enzyme associated with the pathogen of interest, a volatile metabolite, an enzymatic substrate containing the chosen metabolite and a tailored xerogel as an analyte concentrator. One promising alternative to pNP in further studies is the use of a fluorescent volatile metabolite, which will allow to increase the sensitivity of the sensor.



**Fig. 5** Detection of pNP released by *E. coli* and trapped in the xerogel (a) set-up. (b) UV-visible spectrum of pNP- trapped in the xerogel exposed to *E. coli* MOPS buffered culture. (c) Kinetics of trapping of pNP, at 383 nm, in a xerogel exposed to *E. coli* MES buffered culture.

## Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details (protocols). See DOI: 10.1039/b000000x/

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## Supplementary material for Facile and fast detection of bacteria via the detection of exogenous volatile metabolites released by enzymatic hydrolysis.

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### 1. Synthesis of xerogel monoliths

The xerogel monoliths have a parallelepiped shape. They were produced via the sol-gel process. The chemicals, reagent grade, were purchased and used without further purification. Silicon alkoxyde (TMOS and APTES) precursors were mixed with methanol and deionized water according to the following molar ratio (TMOS/APTES)/MeOH/H<sub>2</sub>O: (0.97/0.03)/5/4. The reaction is extremely fast due to the alkaline catalysis of APTES and must be carried out at -20°C. 1mL of this sol was immediately poured into a cuvette for spectrophotometry (Fisherbrand disposable cuvette; semimicro; polystyrene; capacity: 1.5mL) to obtain a monolith with 12mm × 5mm × 2mm size. During the drying process, operated slowly at 22°C in humidified atmosphere (55% relative humidity) during 3 days, the cuvettes were sealed with an adhesive microporous film (ABGene Gas permeable adhesive seals).

### 2. Trapping pNP (p-nitrophenol) in a monolith

*2.1 Exposure to gaseous pNP above an aqueous pNP solution*

pNP, spectrophotometric grade, was obtained from Fluka. An aqueous solution of 0.11M pNP was prepared. A monolith is placed in a small glass vessel (2.6mL), containing 60μL of the pNP solution. The monolith is not in contact with the solution and is exposed to the vapors (pNP and water) over 48h at 25°C. A UV spectrum of the monolith is recorded with a Cary 300 spectrophotometer before and after exposure. The variation of absorbance at 330 and 385 nm witnesses the trapping of pNP in the monolith, under its protonated and deprotonated form.

### *2.2 Exposure to gaseous pNP coming from a bacterial culture*

The culture medium containing the substrate, targeting β-D-glucuronidase, was optimised to increase the glucuronidase activity. It also contained a buffer at pH 7.33, to keep the ratio pNP/pNP<sup>-</sup> constant. The MOPS medium was composed of: MOPS (4-morpholinepropanesulfonic acid sodium salt) 150mM, magnesium sulfate 2.6mM, sodium glucuronate 854μM, methyl β-D-glucuronide 870μM, and 4-nitrophenyl-β-D-glucuronide 113μM. The MES medium has an identical composition, except for the buffer: MOPS 150mM was replaced by MES 150mM (4-morpholineethanesulfonic acid). The resulting pH is 6.1.

After a pre-culture phase overnight (37°C on TSA, Trypcase Soy Agar, bioMérieux), several macro-colonies of *Escherichia coli* ATCC11775 were picked up on TSA and dispersed into Suspension Medium (bioMérieux) to adjust the cell concentration to 0.5McF (measured with a Densicheck, bioMérieux). With the colonies enumeration on agar plates, we measured that the 0.5 McF optical density value corresponds to 10<sup>8</sup> cfu/mL. The enzymatic assay, shown in Figure 5, started at t=0, when the 0.5McF bacteria suspension was diluted (1/500 or 1/1000) with the culture medium containing the enzymatic substrate. The paranitrophenol, released by the microbial culture and present in the gas phase, was trapped in the monolith placed in a spectrophotometric cuvette, under a recipient (Ependorf tip) containing the bacterial culture (see Fig. 5a). The amount of trapped pNP was monitored spectrophotometrically. A first exposure was carried out with *E. coli* inoculated in MOPS medium at 2.10<sup>5</sup> cfu/mL. After 17 hours of incubation, 500μL of the culture were poured in the recipient (Ependorf tip). A cell with a monolith exposed to the laboratory atmosphere was used as a reference (see Fig. 5b). A second exposure was carried out with *E. coli* inoculated in MES at 10<sup>5</sup> cfu/mL. After 9 hours of incubation, 1.5mL of the culture was poured in the recipient. An empty cell was used as a reference. The UV spectrum of the monolith was monitored with a Cary 300 spectrophotometer overnight (see Fig. 5c).

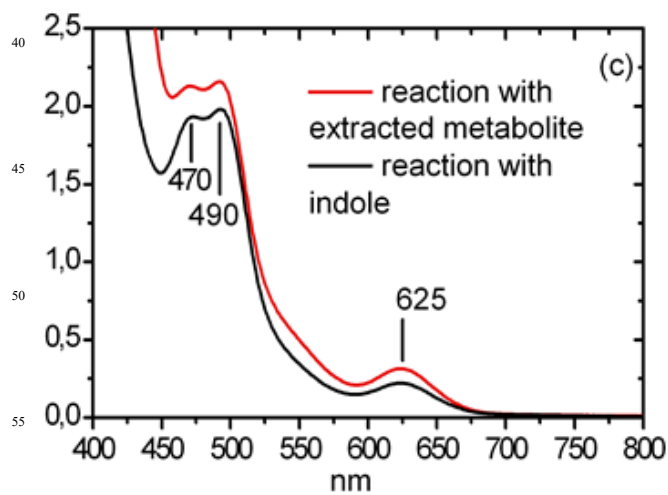
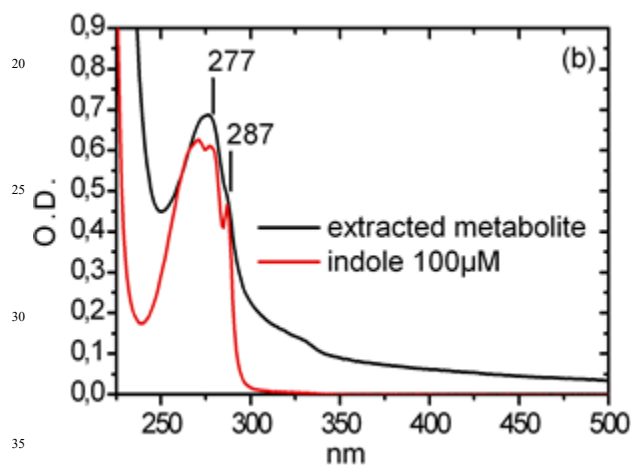
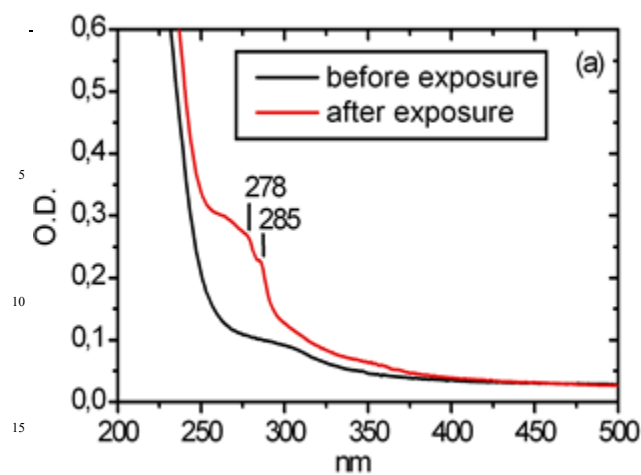
### 3. Control experiment without enzymatic substrate

*3.1 Exposure of a xerogel monolith to a bacterial culture without pNP enzymatic substrate*

After a pre-culture phase overnight (37°C on TSA), several macro-colonies of *Escherichia coli* ATCC11775 were picked up on TSA and dispersed into Suspension Medium, to adjust the cell concentration to 0.5McF. The UV spectrum of the monolith was recorded with a Cary 300 spectrophotometer before and after exposure. The control experiment started at t=0, when the 0.5McF bacteria suspension was diluted (1/1000) with the culture medium non-supplemented with enzymatic substrate. Two xerogels were placed within the headspace of the bacterial culture and the spectrum was collected after 22 hours of exposure (Figure S1 a).

### *3.2 Extraction of trapped indole and reaction with DMACA*

Two xerogels were ground and mixed with 3mL of methanol to extract the trapped volatile metabolite. After 24h of impregnation at room temperature, the solution was filtered (0.2 μm Ø) and the absorption spectrum of the unknown metabolite was collected (Figure S1 b). The hypothesis that the metabolite is indole was based on the fact that the nutrient medium contains aminoacids such as tryptophane, and *E. coli* strains are known to produce tryptophanase, an enzyme that hydrolyses tryptophane into indole. As a proof, the spectrum of a solution of indole was collected and compared to the metabolite spectrum (Figure S1 b).



60 Figure S1. (a) Spectrum of a xerogel monolith collected after 22h of exposure at 37°C to a bacterial culture non-supplemented with enzymatic substrate pNPG. (b) Spectrum of the metabolite in methanol, extracted from ground xerogel. For comparison, the spectrum of a methanolic solution of indole (100 µM) is also plotted. (c) The unknown extracted metabolite is mixed with DMACA under acidic conditions. This reaction is specific to indole. For comparison, the reaction is performed in similar conditions with a methanolic solution of indole.

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### 3.3 Reaction with DMACA

To strengthen the attribution of the metabolite to indole, we achieved the selective condensation of indole with DMACA (4-(dimethylamino)cinnamaldehyde), known to selectively react with indole under acidic conditions to yield the strongly absorbing azafulvenium chloride salt, displaying a high extinction coefficient value at 625 nm ( $\epsilon(625\text{nm}) = 97\,000 \pm 13\,000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ , this work).

The following procedure was applied: 500 µL of the methanolic solution of the extracted metabolite was mixed with 75 µL HCl 37% and 925 µL of DMACA 0.0648 M. The final concentrations of the reactants are: [HCl]=0.6 M and [DMACA]=40 mM. The spectrum collected after the reaction (Figure S1 c) displays the typical absorption band of azafulvenium. As a control, we performed the same DMACA test with 500 µL of a methanolic solution of indole (10 µM). The resulting absorption spectrum (Figure S1 c) being identical to the former one, the unknown volatile metabolite was definitely attributed to indole.

Furthermore, as a blank test, we performed a similar experiment with a culture of bacteria in a medium non-supplemented with pNPG and using a strain that is unable to produce indole. After 22 hours of exposure in similar conditions, both of the xerogels were characterized with UV-vis. Spectrophotometry: no absorption band was found around 280 nm.

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