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New Culture Media for Fast, Sensitive and User-Friendly Detection of *E.Coli* and/or Coliform Bacteria

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Abbreviations: iCFU: initial Colony Forming Units; OD: Optical Density; RPM: Rotation Per Minute; MU: 4-Methylumbelliferone; MUG: 4-Methylumbelliferyl-B-D-Glucuronide; ONPG: Orthonitrophenyl-B-D-Thiogalactoside; ONP: Orthonitrophenyl; B-Gluc: B-Glucuronidase; B-Gal: B-Galactosidase; TTD: Time to Detection; IPTG: Isopropylthiogalactoside

ABSTRACT

Objective: Bacterial contamination of drinking water is a public health concern ruled by national standards and by the world health organization guidelines. Efforts have thus been constantly devoted to improve detection of markers of contamination as early as possible, each time contamination. In this work, we optimize detection of coliform bacteria in general, and of *E. coli* in particular, by modifying specific growth media.

Methods and results: By reinvestigating key parameters such as selectivity, growth kinetics, signal intensity, number of steps in sample manipulation and signal monitoring, we succeeded in i) improving the rate of bacterial contamination detection (medium SMIL), ii) finely tune the ratio of contamination revelation over bacterial growth (medium SMIM), or iii) designing a user-friendly and cost-effective signal detection system (Chromo-SMIM medium).

Conclusions: SMIM and SMIM-derived media present a better β-galactosidase (present in all coliforms) activity detection compared to classical methods. This new method thus is a useful improvement of standard techniques used worldwide in water-control analytical laboratories.

INTRODUCTION

Coliform bacteria, including the *Escherichia coli* (*E.coli*) subgroup, are Gram-negative (Gram-) bacteria commonly found in human and animal faeces [1]. Their presence in drinking water is directly related to faecal contaminations of water. Although most of them are not harmful to humans, their detection may indicate the presence of pathogenic organisms such as other bacteria, protozoans, viruses or other kind of parasites that also live in human and animal digestive tracts. As it is expensive, time-consuming and difficult to test the presence of several pathogens, drinking water samples are tested for coliform bacteria and/or *E. coli* presence. There is a large consensus on the fact that a small amount of coliform bacteria or *E. coli*, i.e. one bacterium in 100 mL of drinking water as defined in the European directive 98/83/EC (3 November 1998), is sufficient to declare water unsuitable for human consumption. Thus, detecting those bacteria is requested to limit the risk of ingesting faecal pathogens, and prevent taste and odours disagreements [2].

Current assays focus on coliforms bacteria and/or *E. coli* because they both present enzymatic characteristics that enable easy identification with different methods [3]. Every coliform bacterium (including *E. coli*) is able to express, under defined

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conditions, β-galactosidase (β-gal), the enzyme that cleaves lactose into glucose and galactose [4]. This ensures access to a readily usable carbon source to the bacterium even in the absence of other sugars. β-galactosidase is coded by the lactose operon, which regulation has been extensively studied [5-10]. Many assays have been developed to reveal a β-galactosidase activity (e.g. lacto-fermentation tests in lauryl tryptone broth or brilliant green lactose bile broth media) [11]. Bacterial detection tests now mostly rely on enzymatic substrates that produce light-based detectable metabolites. For β-galactosidase, for instance, a widely used substrate is orthonitrophenyl-β-d-thiogalactoside (ONPG). ONPG cleavage releases one galactose molecule and one orthonitrophenyl (ONP) dye molecule. Bacteria use galactose as a carbon source and ONP accumulates in the medium as a yellow molecule with maximal absorbance at 420 nm.

In contrast to other coliforms, *E. coli* also expresses β-glucuronidase (β-gluc) in addition to β-gal. This offers the possibility to use another chromogenic molecule that is a specific β-gluc substrate. The most widely used molecule is 4-methylumbelliferyl-β-d-glucuronide (MUG), cleaved into glucuronide (used as a carbon source by the bacterium) and 4-methylumbelliferone (MU), a fluorescent product with an excitation peak at 365 nm and maximal emission at 460 nm.

A commercially available medium (colilert-18® [12,13]) that detects coliforms and discriminates between coliforms (the medium becoming yellow) and *E.coli* (the medium becoming yellow and fluorescent upon excitation with a UV lamp), contains the two substrates for b-Gal and b-Gluc as the sole carbon sources. Non-coliform bacteria will not grow nor produce any signal as they exhibit neither β-Gal nor β-Gluc activity. This medium is used as a standard in several countries and is an alternative to plating methods, which require sample filtration -to concentrate bacteria- and plating on solid media (Grant, 1997). Colilert-like methods require no filtration and are sensitive enough to detect one bacterium in a 100 mL water sample in 18 h.

Several parameters to optimize must be considered to develop media for coliform and/or *E. coli* bacteria detection. Among them is selectivity (to avoid noise arising from non-specific organisms), growth rate (or time to detection), intensity of the signal (sensitivity of the detection), the number of steps in sample manipulations (should be user-friendly, or best unnecessary) and signal monitoring or recording (cost effective detectors).

In this work, we design several media for coliform and/or *E. coli* bacteria detection that address the challenge of detecting one coliform/*E. coli* bacterium in less than 12 h. All media share a common base (referred to as the "base medium"), and thus present similar general characteristics. The specificity of each medium is conferred by the "substrates" that provide carbon sources and detection probes. Each medium has been optimized to fit at least one of the key parameters listed above. Three original media are described that optimize the revelation over growth ratio, improve the rate of detection, and make use of cost effective detection devices

MATERIAL AND METHODS

Colilert was purchased from Idexx®. It is conditioned as a bottled powder to which 100 mL of water sample is added to initiate bacteria detection. Contaminant-free ultrapure grade water (produced on a Z00QSV0WW purifier from Millipore®) was used to ensure repeatability. Media have been inoculated as described below.

The SMIM medium and derivatives are composed of two distinct components, a base medium (containing nutrients and additives) and a set of substrates. Major differences between the media lie in the nature of the substrates. The exhaustive list of all constituents is displayed in **Supplementary Table 1**.

Base Medium

Nutrients are provided in the DMEM medium (without glucose, L-glutamine, Phenol Red, Sodium pyruvate and sodium bicarbonate), purchased from Sigma-Aldrich® as a powder. 8.3 g of nutrients are weighted to prepare 200 mL of 5 x concentrated medium.

All additives except sodium bicarbonate, nicotinic acid, nicotinamide and Tergitol-7 are weighted separately and then pooled together with the base medium powder. 100 mL of ultrapure water are added to dissolve powder. Nicotinic acid and nicotinamide are prepared separately in ultrapure water as 1000x concentrated solutions and added at this step. Liquid Terigtol-7 and sodium bicarbonate powder are then added and the final volume is adjusted to 200 mL to obtain the desired 5x concentrated base medium (with nutrients). The concentrated solution is filtered on a 0.45 µm nitrocellulose filter under sterile environment and stored at 4°C until use.

Substrates are prepared as 400x concentrated DMSO solutions under argon atmosphere to eliminate oxygen. Solutions are stable at room temperature for more than three months in the absence of light. Substrates are added to the base medium/additives mix just before use.

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20 mL of 5x base medium are extemporaneously placed in a 110 mL bottle together with 250 µL of each 400x concentrated substrate. The bottle is filled to 100 mL with the water sample to test. For this study, ultrapure grade water was used to avoid bacterial contamination and ensure repeatability. Media have then been inoculated as described below.

To assess media characteristics, a well-defined amount of bacteria was added at the beginning of each experiment. This starting bacterial quantity is referred to as the Initial Colony Forming Units (iCFU).

The day before experiment, cultures of the selected strain(s) are performed in LB medium, incubated overnight at 35°C and stirred (220 rpm) in the dark. On the day of the experiment, cultures are centrifuged for 5 minutes at 3300g at room temperature (to remove LB) and bacteria are suspended in water. OD600nm is measured to assess bacterial concentration. Cultures are diluted in cascade in 1 mL of water until the desired iCFU per 100 µL is reached. Inoculation is performed with 100 µL of the selected dilution to ensure reproducibility. 100 µL of the same dilution are plated on LB-agar plates and incubated over night at 35°C to assess the exact number of bacteria (iCFU) using the pour plate method. Further dilutions are carried out if the iCFU count is too high (>400 colonies) to reliably determine the initial number of bacteria.

Immediately after inoculation, cultures are incubated at 35°C in the dark without stirring. Optical densities for bacterial count (OD600) or substrate production (OD420) were either measured as end points or in a continuous manner. The blank was measured with the same medium inoculated with 0 bacterium per bottle and submitted to the same treatments.

On-line measurements for OD420 nm were performed automatically using a circulating system from the culture bottle to a circulation quartz cuvette (QS0300, 100 µL), with a Beckman DU640® spectrophotometer. The cuvette was connected to the culture with 3.5mm tubing. Circulation was ensured by a Minipuls2® peristaltic pump from Gilson **(supplementary Figure 1a)**. The flow rate was set at 6.56 mL/min to ensure the circulation of the liquid through the whole system in 4 min and the whole cuvette volume is replaced more than once per second, while avoiding foam formation. The liquid is pumped out from the bottom of the bottle and returns at the top of the bottle to ensure a full circulation. The system presents a two minutes dead time at the beginning of the experiment, the time needed for the liquid to reach the cuvette. After this dead period a measurement is possible every second. In this work, data have been recorded every second for up to 30 minutes after 4000 iCFU inoculation.

Methylomelliferone, MU, signal has been assessed directly in the bottle with a Fluo-sens device (ESMO 33-M4034 ESE laser®) from QIAGEN® **(Supplementatry Figure 1b)**. This is a compact portable fluorescence detector easily disposed on culture flasks. It is in contact with the flask external wall, directly into the incubator. The laser is set to emit light every 6 minutes, at a focal distance equal to 9 mm inside the bottle. The 365 nm-length excitation light, triggers MU fluorescence emission detected at 460 nm. Fluo-sens measures fluorescence with a photomultiplier. Fluorescence values, expressed in arbitrary units, range from 0 to 2500 and the sensitivity is set to the minimum on the FLdigital software (Qiagen®) to set background at a low level (500 a.u for Colilert-18®, 700 a.u. for SMIM and derivatives) and detect signal with the largest possible dynamic range.

For detection in SMIL medium, bacteria are cultivated at 35°C in a 100 mL bottle containing SMIL medium. 100 µL samples are taken up at indicated time and treated 30 minutes at 35°C with 10 µM of Triembarine AM to reveal the presence of the specific grown bacteria. The intensity of fluorescence is read with a fluorescence plate reader EnVision® Multilabel Plate Reader (PerkinElmer). Sample is analysed after the 5 first hours of growth to avoid biasing growth rate by taking up proliferating bacteria.

EC1-31 are *E. coli* water born bacteria purchased from Eurofins®. CT4 and CT5 are total coliforms (non *E. coli*) water born bacteria purchased from Eurofins®. NC8 are Pseudomonas Aeruginosa water born bacteria purchased from Eurofins®. *Bacillus subtilis* used in this study are water born bacteria purchased from Eurofins®. ATCC8739 are *E. coli* bacteria purchased from the American Type Culture Collection®.

Field sample 0 refers to water coming from the laboratory sink. Field samples 1-5 have been collected by Eurofins® at the indicated sites **(Table 1)** after suspicion of contamination in a given area. Field samples 6-7 have been collected by Eurofins® at the indicated sites **(Table 1)** without contamination suspicion. Field samples 8-11 have been randomly picked up at the indicated locations **(Table 1)**.

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Table 1. Bacterial detection in field samples. 100 mL of the indicated field samples have been analysed with SMIM for the presence of total coliforms or *E. coli* bacteria. The most probable number (MPN) of bacteria present in the sample has been assayed by Eurofins® (samples 1-7) and in our lab using Colilert-18® in the Quanti-tray2000® system from Idexx® according to customer recommendations (sample 0-11). <1 means that no well of the quanti-tray plate was positive. Detection by SMIM has been carried out for 18h at 37°C. For total coliforms, the OD420 nm (yellow intensity) has been measured. Symbol (++) stands for OD420nm>0.5; (+++) means OD420 nm>1.5 and (++++) for OD420 nm>2.5. *E. coli* presence is revealed by λ460nm measurement with ESE laser as described above. Symbol (+) indicates a fluorescence intensity >2500. All experiments were repeated three times. Numerical values are means of three experiments.

N°	Sample provenance	MPN/100 mL		SMIM Detection	
		Total Coliforms	E. Coli	Total Coliforms OD420nm	E. coli Fluorescence λ460nm
0	Sink - research institute of biotechnology Superior School - Illkirch France	<1	<1	-	-
1	River "La Mauchère"- Custines France	>2420	>2420	++++	+
2	River "La Meurthe" -downstream from wastewater plant-Nancy France	>2420	>2420	++++	+
3	River "L'Amezule" - Lay St Christophe France	>2420	>2420	++++	+
4	Creek "Le Gremillon"-Essey les Nancy France	>2420	1200	++++	+
5	River "La Meurthe" - Nautic base - Nancy France	>2420	1700	++++	+
6	Upstream from water reservoir - Bellefontaine France	110	<1	++	-
7	Downstream from water reservoir - Bellefontaine France	14	<1	++	-
8	River "La Zorn" -Geudertheim France	145	<1	+++	-
9	River "La Moder" - upstream from wastewater plant-Rohrwiller France	63,7	<1	+++	-
10	River "La Moder" - downstream from wastewater plant- Rohrwiller France	36.8	<1	+++	-
11	Gravel pit - Bischwiller France	20.3	<1	++	-

RESULTS

Media Suitable for 1 Bacterium Detection in 100 mL

In the commercial medium (Colilert-18®), the concentrations of the two substrates, 1,7 mM of ONPG and 0,21 mM of MUG, are such that the enzymes β-galactosidase (K_M of 4.45 x 10^{-4} M for ONPG) and β-glucuronidase (K_M of 0.5 x 10^{-4} M for MUG) work at about 80% of their maximal velocity "V_{MAX}" (79% and 81% respectively). Approximating that the enzymatic reaction rate "V" is equal to V_{MAX} x S/(K_M + S), using a substrate concentration of S=10 x K_M would guarantee a reaction rate equal to 90.9% (10/11) of V_{MAX}. Detection may thus be improved by using a larger concentration of substrate and the amount of ONPG and MUG should be equal to 0.136 g and 0.018 g respectively, for 100 mL of medium. The composition of all media prepared in this study is given in supplementary **Table 1.** As shown in **Figure 1a** escalation of substrate concentration from 1.7 to 2.5 mM for ONPG and from 0.21 to 0.5 mM for MUG (indicated as "+ substrate") improves bacterial growth (OD600 **Figure 1a** left panel) and increases the intensity of the yellow signal of ONP (OD420 **Figure 1a** right panel) 18 h after initial inoculation with 100 *E. coli* bacteria. Adding another carbon source such as glucose further improves growth (**Figure 1a**, left panel) but drastically reduces detection sensitivity as measured by the intensity of the ONP signal (right panel), as a result of preferred glucose consumption over galactose.

In order to take full advantage of enzymatic reaction kinetics, we tried to improve the culture medium for rapid bacterial detection according to the following principles: i) the only carbon source provided is that of β-Gal and/or β-Gluc substrates, and ii) the pH of the medium is set to detect substrates at their highest absorbance wavelength (see material and methods and supplementary **Table S1**). The selected substrate, ONPG and MUG, allows electivity for β-galactosidase (coliforms) and β-glucuronidase (*E. coli*) positive bacterial strains. They are used at the highest reasonable concentration. SMIM is suitable for bacterial growth and ONP signal generation **(Figure 1b)**. Removing substrates from the SMIM composition does not allow bacterial growth and does not develop ONP signal as expected **(Figure 1b)**. The addition of 2% (W/V) D-glucose in SMIM promotes growth but reduces detection level efficacy. Regarding optimal pH we determined ONP signal intensity in SMIM and colilert-18® media at various pH values **(Figure 1c)**. In the two media ONP signal is higher at pH 7.5-8 in Colilert-18® and at pH 10 in SMIM. We determined that the pH of SMIM is close to 8.7 before- and about 8.2 after- bacterial growth. In contrast, Colilert-18® pH varies from 7.8 to 7.3 in the same conditions (data not shown). Clearly, the higher pH of SMIM ensures brighter ONP yellow color development **(Figure 1d)**.

Drinking water standards (European directive 98/83/EC) consider drinking water as contaminated if as few as one coliform bacterium is present in a 100 mL sample volume. Thus, we checked if the limit of detection of SMIM meets this requirement. In a first attempt, we measured growth and ONP signal 18 h post-inoculation in SMIM and in Colilert-18®. **Figure 1d** illustrates that bacterial growth is slightly lower in SMIM than in colilert (OD600 left panel) but that the ONP signal is brighter (OD420 right panel), supporting that the increase in pH, compensates for a slight reduction of bacterial growth.

Thus, we describe a medium (SMIM) derived from DMEM that is able of detecting one bacterium in 100 mL with 20-30% better ONP signal intensity than that of the reference medium.

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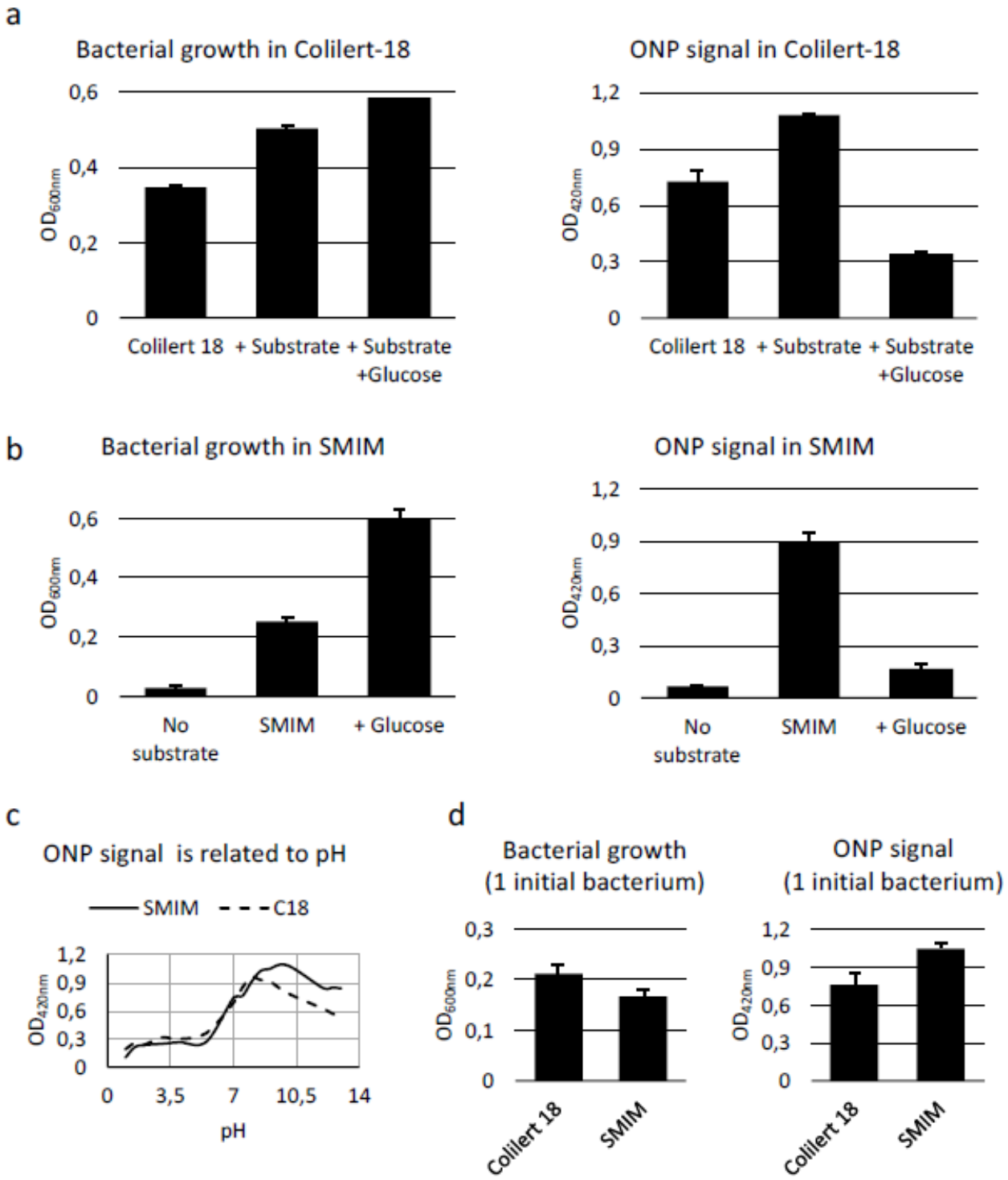

Figure 1. Media suitable for 1 bacterium detection in 100 Ml. a) and b) Incubation of 100 *E. coli* (EC18) bacteria in 100 mL of Colilert-18 or SMIM during 18 h at 37 °C. Then measure of bacterial growth (left panel) and ONP signal (right panel). Results are means of three independent experiences +/- standard deviation. +Substrate indicates that medium has been supplemented with 0,136 g of ONPG and 0,018 g of MUG for a 100 mL test. +Glucose stands for the addition of 2% (W/V) D-glucose to media. c) ONP signal measurement of 18 h at 37 °C grown culture in 100 mL of Colilert-18 or SMIM after variable HCl or NaOH quantities addition to obtain desired pH. d) as a) and b) with a starting concentration of 0,3 bacteria/100 µL resulting in one bacterium per 100 mL bottle.

Real-time fluorescence and absorbance recording

To monitor the appearance of coumarin (the fluorescent metabolite of MUG) in the culture medium, we used a portable laser excitation device equipped with a diode sensor (ESE®). The device is placed directly in contact with the culture bottle and monitors fluorescence inside the culture medium **(Supplementary Figure 1)**. Repeatability of MU signal measurement was assessed using independently inoculated cultures grown in the reference medium Colilert-18® **(Figure 2a)**. Three independent

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inoculations were performed with 100 bacteria in the bottle **(Figure 2a**, red lines) and two with 50 bacteria **(Figure 2a,** left panel, green lines). Fluorescence time courses superimpose perfectly among identical inoculates (identical iCFU), and reach the laser saturation signal of 2500 a.u. at the same time. **Figure 2a** right panel shows the mean of three independent experiments (100 iCFU from the left panel) +/- standard deviation.

Similarly, development of MU fluorescent signal was recorded for different iCFU **(Figure 2b)** in Colilert-18® and SMIM over 18 h **(Figure 2b)**. In this study, we define Time to Detection (TTD) as the time needed for the signal to reach 2.5 times the initial value. The ESE saturates at a signal of 2500 which is over 2.5 times the initial value. TTD is shorter in Colilert-18® than in SMIM at all iCFU tested, indicating that Colilert-18® MU signal appears 0 to 1 hours earlier depending on the iCFU value.

We plotted the time to detection (TTD) as a function of log (iCFU) and observed an identical exponential relationship for both Colilert-18® and SMIM cultures **(Figure 2c)**. The Pearson correlation coefficient (>0.98 for both experiments) indicates the accuracy of the procedure and allows determination of the iCFU of a water sample just by looking at the TTD for a given strain. This also validates the high reproducibility of the method for both SMIM and Colilert-18® media.

We also monitored the development of ONP yellow color over 18 h with a 4000 iCFU inoculate of environmental *E. coli* (EC18) strain in Colilert-18® or SMIM, as well as in mono-SMIMo **(Figure 2d)** which contains only one substrate **(Table 2)**. Mono-SMIMo presents a higher maximum signal than the two other media because bacteria only have access to one carbon source (the ONPG) leading to a higher accumulation of colored product (ONP). The slight signal reduction at the end of the experiment is due to the high bacterial cells accumulation according to OD600 observation (data not shown). In contrast to SMIMo, in SMIM and Colilert-18®, bacteria digest both substrates. The yellow signal in SMIM and mono-SMIMo is detected faster than in the reference medium **(Figure 2d)**.

Table 2. Media specificities. Colours indicate the colour of the medium after detection. λ460 nm and λ510 nm indicates that media are fluorescent after detection and emits fluorescence at the indicated wavelength after excitation with a UV lamp. Mono-SMIMo and SMIL do not allow distinction between Total coliforms (TC) and *E. coli* (EC). Mono-SMIMm and Mono-SMIMx do not detect non-*E. coli* coliforms (Total coliforms). Base medium alone does not allow any bacterial detection.

	Substrates and reagents						Detection	
Base medium	**ONPG**	**MUG**	**X-gluc**	**Lactose**	**Triembarine**	**Total coliforms (TC)**	***E. coli* (EC)**	**Distinction TC-EC**
SMIM	x	x					λ460 nm	x
Chromo-SMIM	x		x					x
Mono-SMIMo	x							
Mono-SMIMm		x					λ460 nm	N/A
Mono-SMIMx			x					N/A
SMIL				x	x	λ510nm	λ510 nm	

We may conclude from these experiments that the metabolic balance in the new base medium favours the consumption of β-Gal substrates (present in all coliforms including *E. coli*) over that of β-Gluc ones (only present in *E. coli*). This is likely due to the overexpression of the lactose operon (including β-Gal expression from *lacZ* gene) induced by the addition of Isopropylthiogalactoside (IPTG, data not shown).

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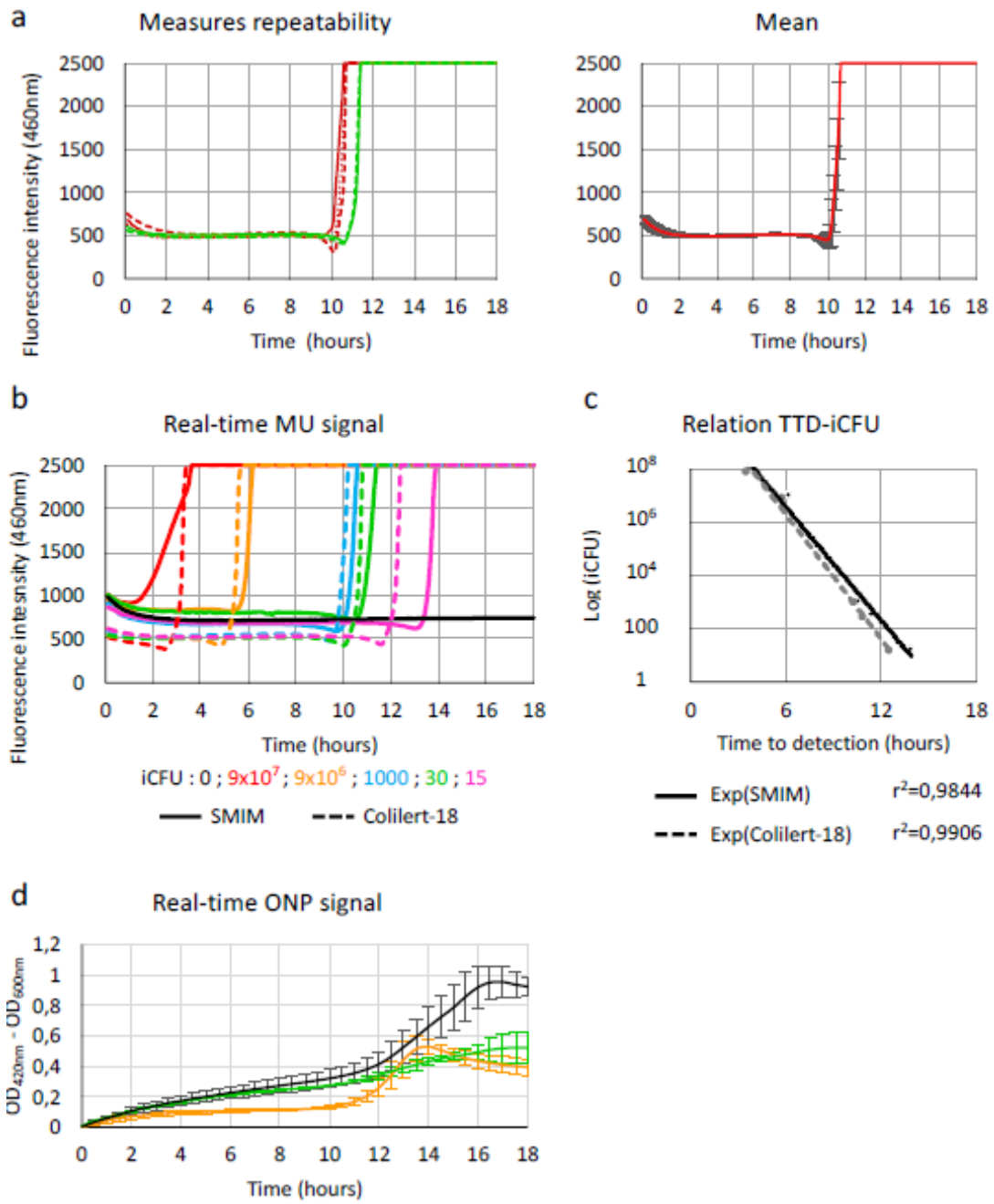

Figure 2. Real-time MU and ONP signals measurements. a) Incubation of 500 (red) or 50 (green) *E. coli* (EC18) bacteria in 100 mL of Colilert-18 during 18h at 37 °C. MU emission at 460 nm by the culture is measured every 6 minutes after excitation at 365 nm by the ESE-laser. Equivalent amounts of bacteria have been tested to assess system reproducibility. Individual data are plotted (left panel) or mean of results obtained for red measurements +/- standard deviation (right panel). The signal saturates at 2500 units. b) MU signal in Colilert-18 and SMIM has been assessed as in a) for different initial colony forming units (iCFU) as indicated. c) The time needed to obtain 2,5 times the initial signal (time to detection or TTD) has been plotted versus the log of iCFU. Exponential regression correlation has been calculated for each media. The experimental curve fitted equations are $y=(7 \times 10^{10})\ e^{-1,635x}$ for SMIM and $y=(8 \times 10^{10})\ e^{-1,789x}$ for Colilert-18®. d) Real-time ONP signal has been recorded (see material and methods), inoculation with 4000 CFU. Results are means of 3 independent experiments +/- standard deviation.

Culture medium specificity for bacterial strains of different environmental origin

We tested the standard medium (Colilert-18®) on different environmental strains (purchased from Eurofins® and described in [14]) to evaluate its selectivity among environmental coliforms and *E. coli* bacteria. In parallel, we tested SMIM for the same set of bacterial strains. **Figure 3a** shows that both culture media are suitable to detect water born bacteria *E. coli* (EC1 to EC31 and ATCC8739) and 2 non *E. coli* coliforms strains (CT4 and CT5)] after 18 h culture as shown by strong growth (OD600 nm) signal. Two non-coliforms Gram- (NC8) and Gram+ (*B. Subtilis*) strains (from environmental origin) do not grow in the media due to their

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inability to access to carbon sources detected by MUG and ONPG. In summary, both media are selective for environmental *E. coli* and coliform over non coliform strains. Colilert-18® better promotes bacterial growth while SMIM significantly favors the ONP signal (OD420 nm) as seen by a nearly 2-fold brighter signal for each of the 32 tested and detected *E. coli* strains and 2 coliform strains (CT4, CT5) as shown in **Figure 3b**. This observation thus generalizes the observation made using strain EC18 **(Figure 1C)**. We thus used the SMIM medium to estimate the presence or absence of total coliforms and *E. coli* bacteria in field samples **(Table 1)**. SMIM allows detection of total coliforms in contaminated samples (table 1, sample 1-11) and of *E. coli* in environmental samples suspected for an outbreak of *E. coli* contamination (samples 1-5). Field samples 1-7 have been collected and validated for the presence of coliforms/*E. coli* by Eurofins®. Field samples 8-11 have been collected by our own and validated using the Colilert-18® Quanti-tray2000® method.

SMIM is thus a sensitive and selective growth medium that outperforms the reference medium for environmental *E. coli* and total coliforms detection.

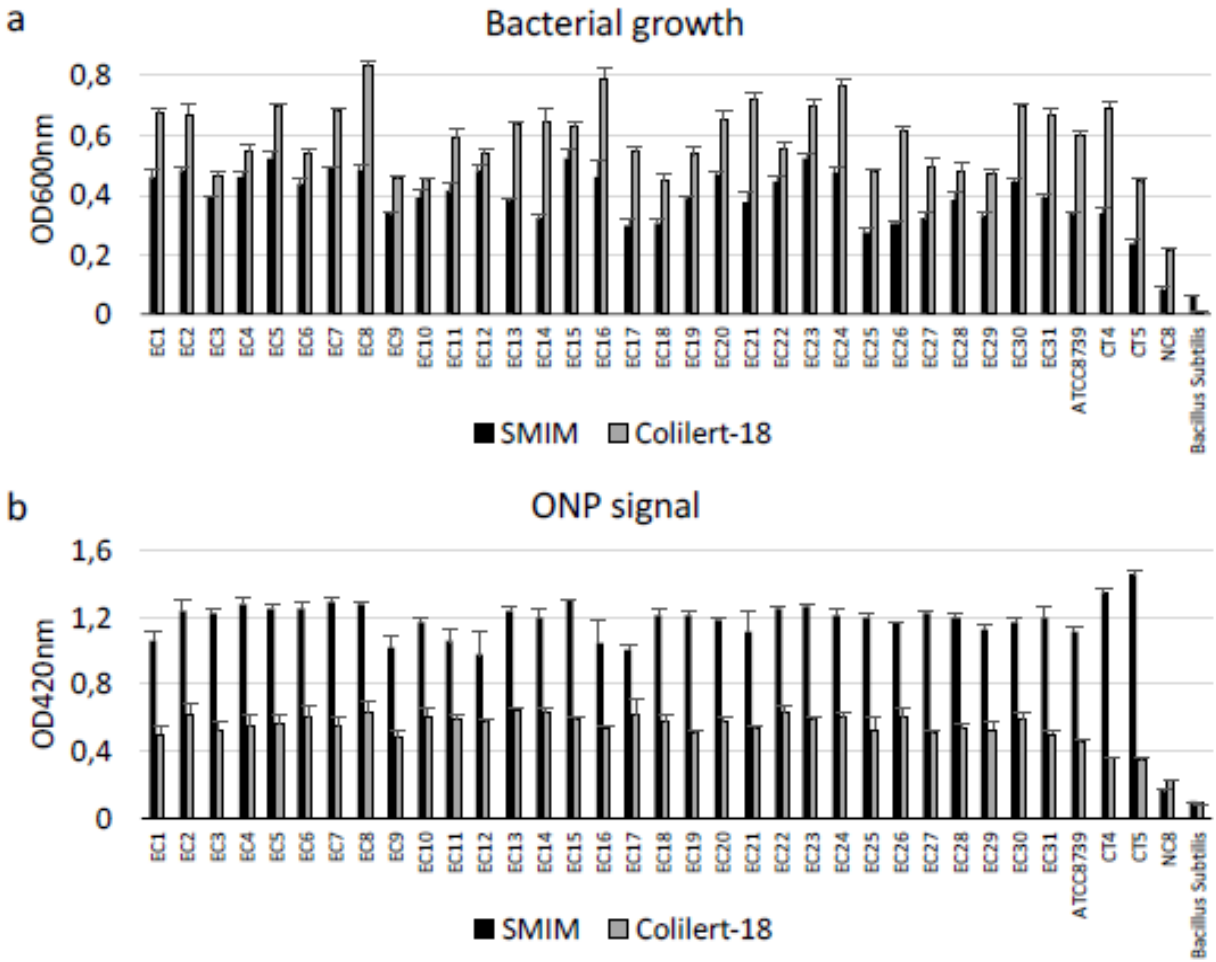

Figure 3. Growth and detection of bacteria from environmental origin Incubation of 500 bacteria in 100 mL of Colilert-18 or SMIM during 18h at 37 °C. 31 water born *E. coli* (EC1-31), 1 databank *E. coli* (ATCC8739), 2 non *E. coli* waterborne coliforms (CT4 and 5), one non-coliform gram- (*Pseudomonas aeruginosa*) and 1 gram+ (*Bacillus subtilis*) strains have been incubated. a) growth (OD600 nm) and b) ONP signal (OD420 nm) and growth have been measured. Results are means of three independent experiences +/-standard deviation.

SMIM Derived Media for Cost Effective Detection

As β-Gal and β-Gluc substrates are important for enzyme selectivity and thus strain selectivity (in addition to antibiotics, antifungals and growth-limiting molecules), we further explored the relationship between substrate nature and quality of *E. coli* and coliform strain detection. One part of the substrate must be a sugar sustaining bacterial growth, while the other part is meant to become detectable as a result of enzymatic cleavage. Many colored substrates have been developed to test different enzymatic activities and to allow detection by different biophysical means.

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SMIM has been modified into different mono or bi-color media by switching the MUG for a different substrate not requiring fluorescence detectors. This allows cheaper detection since fluorescence detectors are expensive. Chromo-SMIM uses two substrates, ONPG and X-gluc **(Supplementary Table 1)**. X-gluc is cleaved by β-glucuronidase to produce a poorly soluble blue molecule (5-chloro, 4-bromo, indolyl). Culture medium density enables the blue dye to remain suspended in the solution during more than 24 hours and manual shaking of the bottle is sufficient to resuspend the precipitate (data not shown). *E.coli* incubation in chromo-SMIM results in a green stained medium while inoculation with coliform bacteria results in a yellow color culture **(Figure 4b)**. The green medium exhibits two light absorption peaks at 420 nm (yellow) for ONP and at 650 nm (blue) for 5-chloro,4-bromo,indolyl **(Figure 4a)**. Total coliform bacteria only exhibit βgal activity that causes the appearance of the peak at 420 nm **(Figure 4a)** and no β-glucuronidase activity is detected **(Figure 4b)**. A second culture medium, mono-SMIMx **(Supplementary Table 1)** containing only x-Gluc, has been used to confirm the broad 650 nm absorption peak of 5-chloro,4-bromo,indolyl **(Figure 4a)** developing the blue staining **(Figure 4b)**.

Two additional media referred to as Mono-SMIM media have been designed to incorporate only one substrate. This reduces the carbon source to a single molecule, improves the sensitivity of the detection of the selected dye, and consequently reduces the time needed to detect the desired bacterial strain. For instance, mono-SMIMo described above (with ONPG only) detects total coliforms but does not identify *E. Coli*. The two other media, mono-SMIMx (with X-gluc only) or Mono-SMIMm (with MUG only), identify *E. Coli* but not the other coliform strains. Such media can be advantageously used to get strong and rapid strain identification. This is illustrated in **Figure 4c**, which shows that MUG fluorescence appears four hours earlier if mono-SMIMm is used instead of SMIM.

In conclusion, using one substrate medium results in faster and more specific bacterial detection but requires parallelisation of multiple tests to get rapid strain identification.

SMIM, Chromo-SMIM and Mono-SMIM **(Table 2)** are based on the principle that the substrate (which determines specificity of the culture medium) leads to the detection when cleaved by specific enzymes. The SMIL medium uses lactose, the natural substrate of β-Gal, indicating that coliforms and *E. coli* bacteria are able to grow in SMIL, but other organisms are not. β-Gal-induced lactose cleavage releases two sugar (glucose and galactose) in a single enzymatic reaction enabling faster growth. Since no colorimetric or fluorescent molecule is released after lactose cleavage by β-Gal, SMIL requires a bacterial detection system.

Triembarine acetoxymethylester (Triembarine AM) is a pro-fluorophore able to penetrate into dead and live bacteria. Esterases from living bacteria, but not from dead ones, remove acetoxymethylester groups from Triembarine AM. This enables Triembarine to become fluorescent and at the same time be trapped inside the cell, leading to accumulation, and thus signal amplification. This molecule exhibits low signal background and high bacterial penetrance capacity [14]. To reveal the presence of living bacteria grown in SMIL, Triembarine AM is added to the medium 30' before fluorescence reading. SMIL allows the detection of about 106 bacteria after 30 minutes incubation with Triembarine AM (data not shown).

Thus, we decided to measure the time needed to detect a single bacterium with SMIL. For that, 1 single bacterium was incubated in 100 mL of SMIL medium, incubated at 35°C, and 100 µL samples were treated with Triembarine AM every hour and fluorescence measured (see material and methods). We observed that SMIL can unambiguously reveal the presence of 1 *E. coli* bacterium after 11 h of incubation **(Figure 4d)**.

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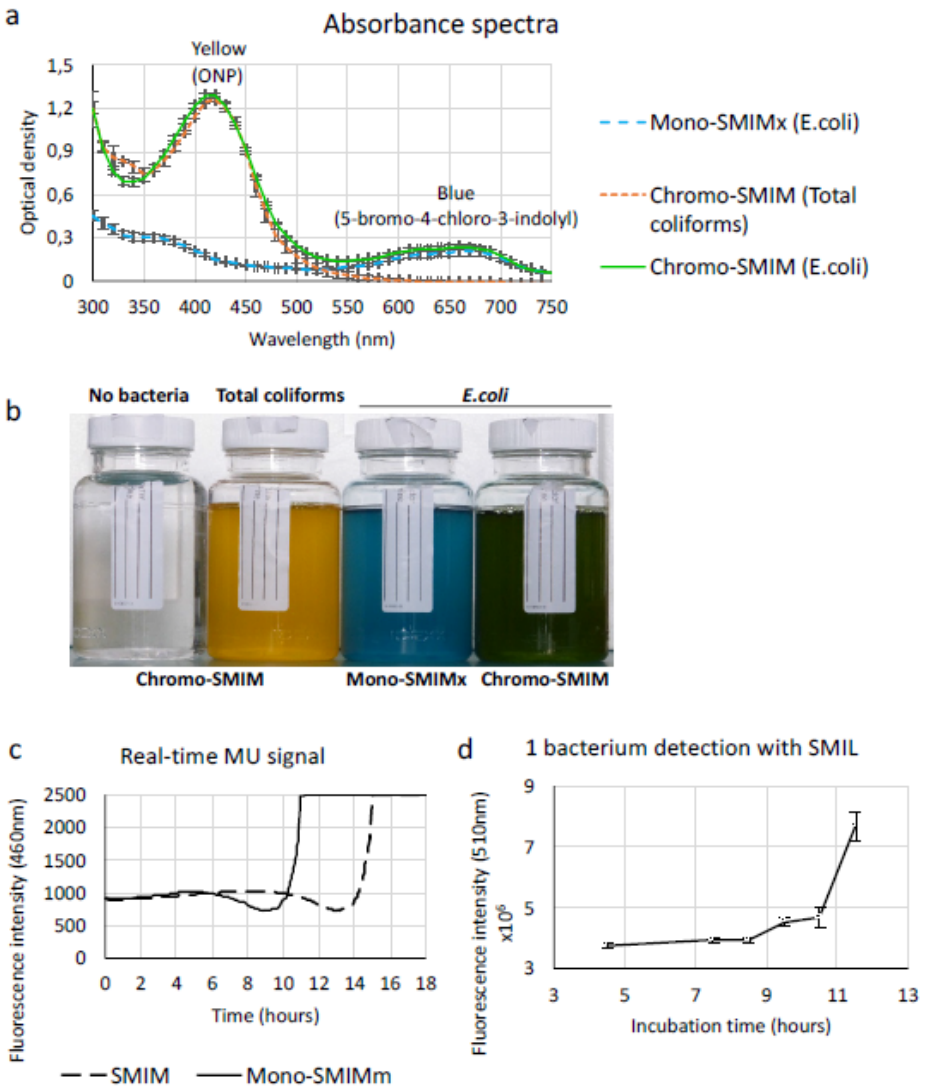

Figure 4. SMIM derived media. a) Absorption spectra of Chromo-SMIM or Mono-SMIMx media inoculated with 100 EC18 (*E. coli*) or CT4 (total coliforms) bacteria after 18 h incubation at 35°C. b) Photographs showing colours of indicated media in a) after the 18 h incubation. c) Fluorescence intensity over time emitted at 460 nm by 10 initial bacteria incubated in SMIM or Mono-SMIMm. d) Detection of 1 bacterium with SMIL. Results are mean of 3 independent bottles +/- standard deviation.

DISCUSSION

In this work we develop new media to detect contaminating coliform and *E. coli* strains in environmental and drinking water samples. Each medium is a compromise between bacterial growth rate and detection of coliforms and/or *E. coli* strains, and costs of the detection process.

Selectivity of the Detection

High selectivity of a medium is desired in order to assign pathogen identity with high confidence. Single substrate media (with the right combination of antibiotics, antifungals and growth limiting molecules) are exquisitely selective as only one subgroup of bacteria will grow. As a consequence, only one bacterial subgroup will be detected ignoring all other species present in the sample. Although single substrate media might be considered as too specific, they advantageously offer a short detection time and an intense signal. On the other hand, detection of multiple contaminants requires multiple parallel assays. Having a complete screen of the bacterial population living in the sample will be easier with multiple substrates media as developed in the Chromocult® assay [15] that is able to discriminate between multiple bacterial subgroups at once without needing any specific detector. It is however slow and requires sample preparation. SMIM and Chromo-SMIM are good compromises enabling easy discrimination between the absence of coliforms, the presence of total coliforms or specifically the presence of *E. coli* only.

Velocity of the Detection

In terms of public health, “Faster is the detection, better the test is”. However, from a technical point of view some methods

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will require sophisticated detectors, which may be too expensive for general use. Manual systems where data recording is human-activity dependent will not gain to be 1 or 2 hours faster as there always will be a minimum gap of 12 h between the time a worker leaves and comes back to work. Automated devices are the best way to make faster detection media profitable but require costlier apparatus as is the case with Colifast® method. Again, the balance between the need and the gain will depend on the expectations. Standard and new media can thus be classified according to the time needed to detect 1 bacterium in 100 mL **(supplementary Figure 2)**. Mono-SMIMm and SMIL-X media are optimized for a fast detection of a single bacterial subgroup.

Intensity of the detection signal

The intensity of the signal in the medium may be critical for samples that present high autofluorescence background, or with very contaminated samples (which may present high density, and/or quenching molecules). The intensity of the signal is also very important for data recording as the sensitivity of the device may vary. The human eye is the data recording device for some media, so it is better to avoid mid-stained media that may lead to controversial decisions. Since every data-recording device (including human eyes) have limit of detection, they also have saturation points and raising the signal over this threshold will not be accompanied by a benefit for the decision. The intensity of β-galactosidase substrates signal (for coliforms and *E. coli*) in each media described in this work (SMIM, Chromo-SMIM, Mono-SMIM) is stronger than in Colilert-18® medium. However, β-glucuronidase signal is a little on the downside (in double substrate medium only e.g. SMIM) compared to Colilert-18. This however is not an issue as MU signal at the end of the experiments is largely over ESE laser saturation point. Furthermore, *E. coli* detection does not only rely on β-glucuronidase activity detection but also on β-galactosidase activity.

Sample Treatment

Treatments required for sample testing may be of importance: First of all, if samples require specific manipulations, specialized workers are required. Testing a sample must involve easy steps, such as adding water sample to a lyophilized medium that is the easiest method for on-field experiments. Moreover, lyophilized media are the best options for product conservation (MUG and ONPG are only stable in the dark, in oxygen-free solution, data not shown). In contrast, automated devices will prefer liquid concentrated media to dispense the appropriate amounts, but this requires (1) concentrated solutions, (2) additives in a separate tank (because they are unstable in the medium), and (3) addition of the water sample. Filtration methods (e.g. Chromocult®) require a filtering apparatus and are time-consuming. Polymerase Chain Reaction (PCR) based techniques are considered as the most selective, faster and more sensitive methods, but require sample treatments (DNA extraction) requiring extreme care, complex protocol, specialized worker and cannot be performed easily on-field [11,16-19].

Data Recording

Data recording can be achieved qualitatively by watching a bottle (which is the easiest way) or by automated device that directly give a quantified result that can be sent without the need of any human intervention. The biggest difference between these two methods is their price that may vary from extremely cost-effective to very expensive. Colilert-18® and SMIM are intermediate media that can be directly observed by human eye for ONP color, but require a UV lamp (or ESE laser for automated recording) using MU signal. Automated (e.g. Colifast®) or semi-automated (e.g. SMIM with ESE laser) recording device have the advantage to be able to trigger alarms before the end of the test as soon as signal reaches a threshold. Chromo-SMIM is the most convenient as it does not requires any device to discriminate between coliform and *E. coli* bacteria.

Impact and Perspectives

SMIM and SMIM-derivate media present a better β-galactosidase (present in all coliforms) activity detection compared to classical methods. Chromo-SMIM does not require any device to read the result except the human eye. Signals in these media can be followed in a real-time fashion using specified devices that allow automatic alarm triggering for rapid water distribution arrest in case of faecal contamination. SMIL-X appears to be well suited for rapid detection of contamination.

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