phss
over 25 years of advancing pharmaceutical and healthcare sciences

European Journal of Parenteral & Pharmaceutical Sciences

Determining incubation regime and time to results for automated rapid microbiology EM methods

Andrew Sage, Nadine Timas and David Jones

Rapid Micro Biosystems, Inc., Bedford, Massachusetts, USA

Reprinted from *European Journal of Parenteral & Pharmaceutical Sciences* 2014
Volume 19 Number 2 Pages 45–55

European Journal of Parenteral & Pharmaceutical Sciences 2014; **19**(2): 45-55
2014 Pharmaceutical and Healthcare Sciences Society

Determining incubation regime and time to results for automated rapid microbiology EM methods

Andrew Sage*, Nadine Timas and David Jones
Rapid Micro Biosystems, Inc., Bedford, Massachusetts, USA

The use of growth-based rapid microbiology methods (RMMs) requires a time to result (TTR) to be determined with a defined incubation regime in order to obtain accurate results and take full advantage of potential time-savings provided by the RMM. A case study involving an environmental monitoring (EM) application to illustrate the simple process was performed using the automated Growth Direct™ System. Recovery of a suite of in-house bacterial and mould isolates was examined at different incubation profiles to define the optimal regime to obtain the best recovery. Of the three, serial incubation at 22.5°C followed by 32.5°C was identified as optimal for the recovery of both the bacteria and mould. A TTR of 72 h for this incubation profile was calculated, and the accuracy of the TTR was confirmed by comparison of the Growth Direct result with spread controls of the test organisms followed by equivalence testing versus the standard method using EM samples. An alternative regime of a single temperature of 28°C was subsequently examined, and resulted in a 60 h TTR, and comparable recovery versus the control spread plates indicating that this may be a viable alternative to serial incubation.

Key words: Time to results, TTR, rapid microbiology methods, growth-based detection, Growth Direct System.

Introduction

Microbial testing in the pharmaceutical industry serves a critical role in product safety by ensuring that manufacturing environments, production processes, materials and finished product meet specified limits of control. In the case of environmental monitoring (EM), microbial testing of air, surfaces, personnel and water is constantly performed to confirm that microbial contamination is maintained within defined acceptable limits[1]; while bioburden testing confirms that raw and in-process materials, as well as finished products, meet specified limits for microbial bioburden.

Testing regimes for EM, bioburden and sterility testing are described in the compendia and other guidance documents[2–4]. Either 30–35°C or 20–25°C may be specified as the standard temperatures to be used. Incubation times can vary depending on the application, but normally range from 3–7 days for bioburden and up to 14 days for sterility. EM testing parameters are also defined similarly with serial incubation times of 3–5 days at 22.5°C and 2–3 days at 32.5°C in order to provide for the recovery of both fungal species (yeast and moulds) and bacteria[1].

***Corresponding author:** Andrew Sage, Rapid Micro Biosystems, Inc., 1 Oak Park Drive, Bedford, MA 01730, USA; Tel: +1 781 271 1444 X234; Fax: +1 781 271 9905; Email: asage@rapidmicrobio.com

While these incubation profiles are defined as appropriate for allowing microbial contaminants to develop into visible colonies, other alternative testing regimes can be used, especially in EM testing wherein serial incubation at the two temperatures can be replaced with a single incubation at an intermediate temperature, for example 28°C. This temperature presumably would allow both moulds and bacteria to develop and provide comparable results while reducing labour and resource requirements.

The primary goal in the use of rapid microbiology methods (RMMs) is to reduce the incubation times specified by standard methods in order to provide a faster time to result (TTR). The shorter TTR of the RMM provides the data needed to enable any necessary action on the sample sooner than the standard method, whether it be product release or to confirm that critical processes are operating within microbiological specification. An important component in the implementation of a newly acquired RMM is the selection of the appropriate TTR for a testing application. Such a determination will balance the requirement for accuracy with the need to obtain test results as fast as possible. An excessively long TTR may lessen any advantages provided by the use of an RMM, and can call into question the rationale for its use if time-savings are the primary goal. Too short a TTR can lead to an inaccurate result wherein contamination may not be detected, thus endangering the process or product.

The TTR determination is specifically needed for growth-based RMMs. Technologies that utilise real-time detection methods, such as epifluorescence or other viability marker-dependent technologies, have a very short time to detection[5], however, such tests do not allow for recovery of the contaminating microbes and have more involved validation requirements[6]. Longer growth-based methods, on the other hand, are non-destructive, thus leaving a viable colony that can then be further characterised and identified as part of a facility's contamination control programme, the importance of which is nowadays emphasised by regulatory bodies[4].

Here, we describe a simple method for determining the TTR for growth-based RMM, and illustrate the process with a case study involving the selection of a TTR for EM monitoring by choosing the optimal incubation profile followed by determining the TTR on an RMM at the selected profile. Additionally, the use of an alternative incubation temperature will be examined as a possible strategy to replace a standard incubation profile. Such a change to single temperature incubation may result in significant resource savings.

Experimental design strategy for setting TTR

A general process flow for a study to determine a TTR for an RMM is outlined as follows. The first step is to define the test protocol or method, followed by the selection of test organisms or samples. The protocol will delineate the specifics of the testing regimes that should be followed in order to generate the data needed to select the TTR, inclusive of growth conditions (media, temperatures, etc). The testing regime consists of the growth of test organisms under the stipulated incubation profile, with the detection of these colonies by the RMM at specific times during the incubation period. At the end of the test, the counts detected per time-point will be plotted as a percentage of the final count obtained by the RMM. The presumptive TTR can then be selected as the time-point at which the RMM result meets a pre-defined acceptance criteria (e.g. > 85% of the final count obtained by the RMM). The counts detected at the TTR will be confirmed by comparison to spread plate controls, and subsequent method suitability testing.

In selecting test organisms, it is important to select strains that have been isolated from the process or environment for which the test is to be applied. Actual process samples may be used as well, however, in most cases, bioburden is too low or non-existent in such samples, thus making it difficult to generate sufficient data for a TTR determination. Also, process samples are often difficult or impossible to obtain in amounts needed for the testing. Another drawback is that bioburden from particular test samples may not necessarily reflect the range of possible contaminants that may be detected at any given time. For example, in EM testing, seasonal changes in the numbers and types of strains detected can occur over the course of a year. In-house isolates, however, allow a wider range of organisms to be tested than would be expected to be found at any one time in actual process samples. Also, such isolates can be subjected to stress associated with a process more easily. For example, if a test material

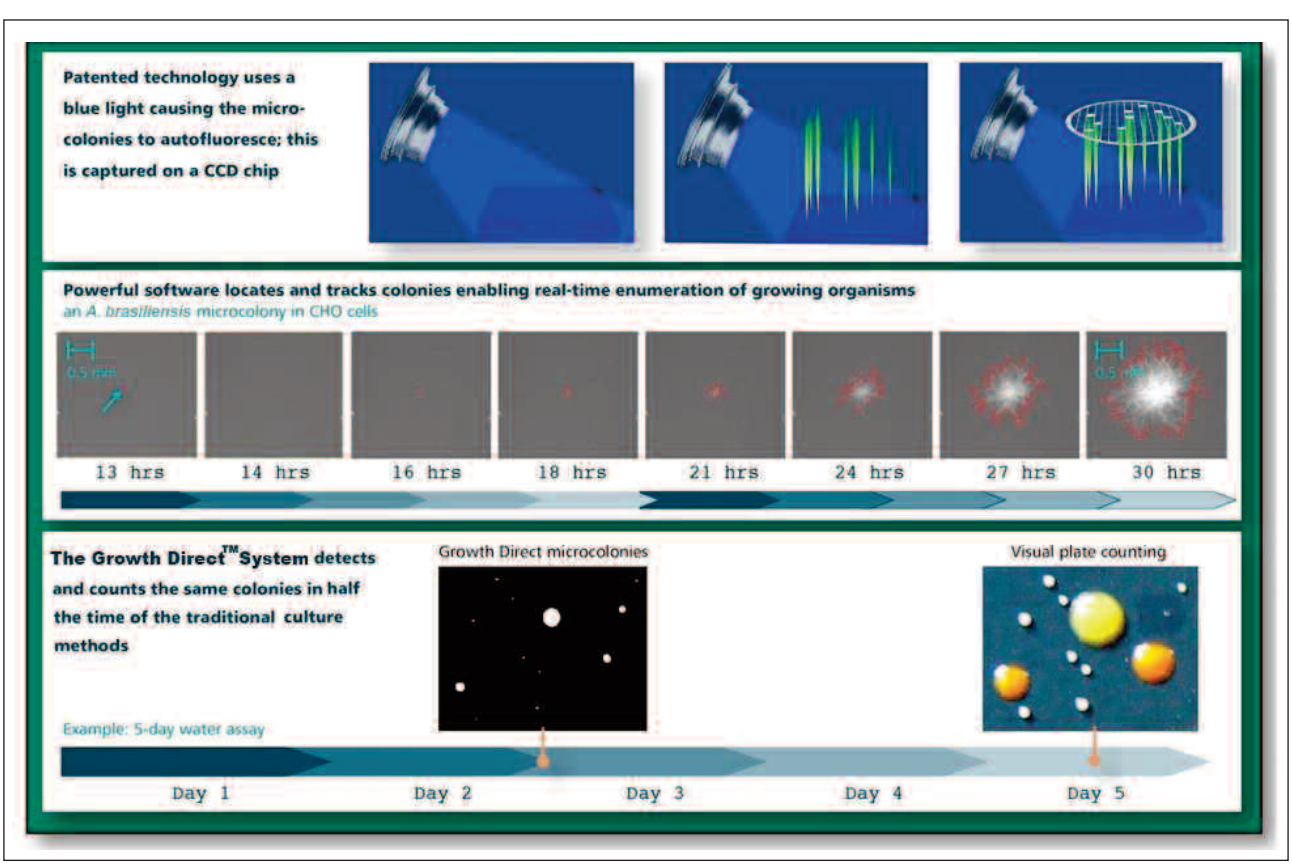

Figure 1. The Growth Direct technology.

contains a low pH, then the test organisms may be subjected to acid treatment to mimic the actual environmental conditions present.

Case study: determining optimal incubation regime and TTR for EM testing on an automated, growth-based RMM

To illustrate the process of selecting a TTR for an application on an RMM, a case study is presented that describes the selection of an optimal incubation regime followed by the determination of a TTR for an EM testing application. The process for selecting the incubation regime was recently described by Moldenhauer[7]. The RMM used in this study was the Growth Direct™ System, an automated, growth-based detection technology[8].

The Growth Direct System uses principles and procedures comparable to the compendial method, including the same media and sampling methods as current testing techniques, and requires little more validation than is currently performed for the compendial methods (**Figure 1**). Its detection technology exploits the fact that all microbial cells emit yellow-green fluorescence when illuminated by blue light due to the presence of fluorescent biomolecules inclusive of (ribo)flavins and flavoproteins (**Figure 1**, top)[9–11]. Like ATP, these molecules are ubiquitous in living systems in all the kingdoms of life including microorganisms[12,13]. By tracking the autofluorescent signal over time, the Growth Direct System replaces the visual plate counting method with digital imaging that detects microscopic colonies before they become visible, thus reducing the time to detection (**Figure 1**, middle). Additionally, the imaging method does not harm the cells and is thus nondestructive, so any micro-colonies present will grow into visible colonies for characterisation after completion of the assay (**Figure 1**, bottom). The user simply has to collect the samples on the Growth Cassette™ and load them into the Growth Direct System. Sample-handling, incubation, analysis and results reporting are fully automated and capable of processing and analysing several hundred samples at a time.

Method

For determining the incubation profile for bioburden recovery and the TTR, the growth of pure culture microbial populations was tracked at measured times using the Growth Direct System. To define the optimal incubation regime, the recovery of the organisms was tracked to completion at three incubation profiles. The profile that supported the best recovery was chosen for the TTR determination. The TTR was then selected based on the time-point at which > 85% of the total counts for each organism was detected compared to the final count obtained on the system. The system count was chosen versus the final visual count from the cassettes as this value is more accurate than a visual count and results in a stricter TTR determination. The selected TTR was initially confirmed by comparison of the Growth Direct System counts with spread plate controls of each organism, and then subsequent comparability testing of EM samples using the Growth Direct System versus the standard method.

The in-house isolates used consisted of both bacterial and mould species isolated from previous EM testing. The species selected exhibited both rapid and slow growth, and were representative of the overall population of strains present. All the isolates had been identified by 16S rRNA sequence analysis to at least genus level (**Table 1**).

Preparation of bacterial growth for testing was performed in the following manner. Test strains were streaked and grown on trypticase soy agar media at either 32.5°C for bacteria or 22.5°C for mould. Sufficient bacterial growth was suspended in trypticase soy broth until visibly turbid, and the absorbance at 600 nm determined. Based on the absorbance, the concentration of colony forming units (CFU) was then estimated. Serial

Table 1. Bacterial and mould strains used in this study.

Bacteria	
Acinetobacter radioresistans	*Pseudomonas putida*
Bacillus circulans	*Roseomonas mucosa* (human)
Gordonia terrea	*Sphingomonas* sp.
Kocuria sp.	*Streptomyces* sp.
Lysinibacillus fusiform	*Staphylococcus caprea*
Micrococcus luteus	*Staphylococcus epidermidis*
Mould	
Aspergillus fumigatus	*Penicillium brevicompactum*
Cladosporidium halotolerans	*Penicillium chrysogenum*
Erwinia rostatum	

dilutions were then performed in phosphate buffered saline to obtain a concentration of 20–200 CFU per 100 μL of sample. The test suspension was then spread onto a membrane filter on an EM Growth Cassette and loaded into the Growth Direct System. For mould species, spore preparations made from sporulated colonies were spread onto the Growth Cassette and then subsequently loaded and analysed by the Growth Direct System.

Testing to define the optimal incubation temperature for the Growth Direct System was carried out at three different temperature profiles to obtain the best recovery of each strain and the shortest TTR. These consisted of incubation at either 22.5°C or 32.5°C for 5 days, or serial incubation for 48 h at 22.5°C followed by 32.5°C for 72 h. This profile was chosen in order to allow temperature-sensitive mould species to develop prior to a higher shift to 32.5°C. The 2-day 22.5°C time-frame for the serial incubation was selected based on preliminary work using two temperature-sensitive mould species, *Penicillium brevicompactum* and *Cladosporium halotolerans*, that resulted in full recovery within 2 days at this temperature. Testing at the alternative incubation temperature of 28°C followed the same procedure.

Analysis of detectable colonies was performed at 4-h intervals by the Growth Direct System. The number of colonies detected and counted at each time-point was then plotted versus the time of the assay. In some cases, overgrowth of the samples led to early termination of the assay; these instances are apparent in the plots of the colony detection curves. The TTR was selected as the time-point at which > 85% of the final count of colonies enumerated by the Growth Direct System were detected.

The presumptive TTR was initially confirmed by comparison of the mean count of each species using the Growth Direct with the side-by-side spread plate controls. Equivalence was defined as a Growth Direct count that was ≥ 70% of the mean control count. Subsequently, equivalence testing against the standard method was carried out with actual samples. Side-by-side air samples were taken on standard contact plates and Growth Cassettes. The contact plates were incubated using the standard parameters of 22.5°C for 3 days followed by 32.5°C for 2 days. Samples tested by the Growth Direct System were incubated at the selected incubation profile for the time specified by the TTR.

Results

Optimal incubation profile for detection of bacteria and mould species by the Growth Direct System

Figures 2 and **3** present plots of the CFU detected versus the incubation time for the test organisms at the three temperature profiles selected for comparison: 32.5°C or 22.5°C for 5 days, and serial incubation at 22.5°C for 2 days followed by 32.5°C for 3 days. **Figure 2** presents curves for bacteria and **Figure 3** for the moulds. A plateau in the colony detection curve for each organism indicated that all colonies present had been detected. As expected, bacterial species exhibited optimal growth at 32.5°C with

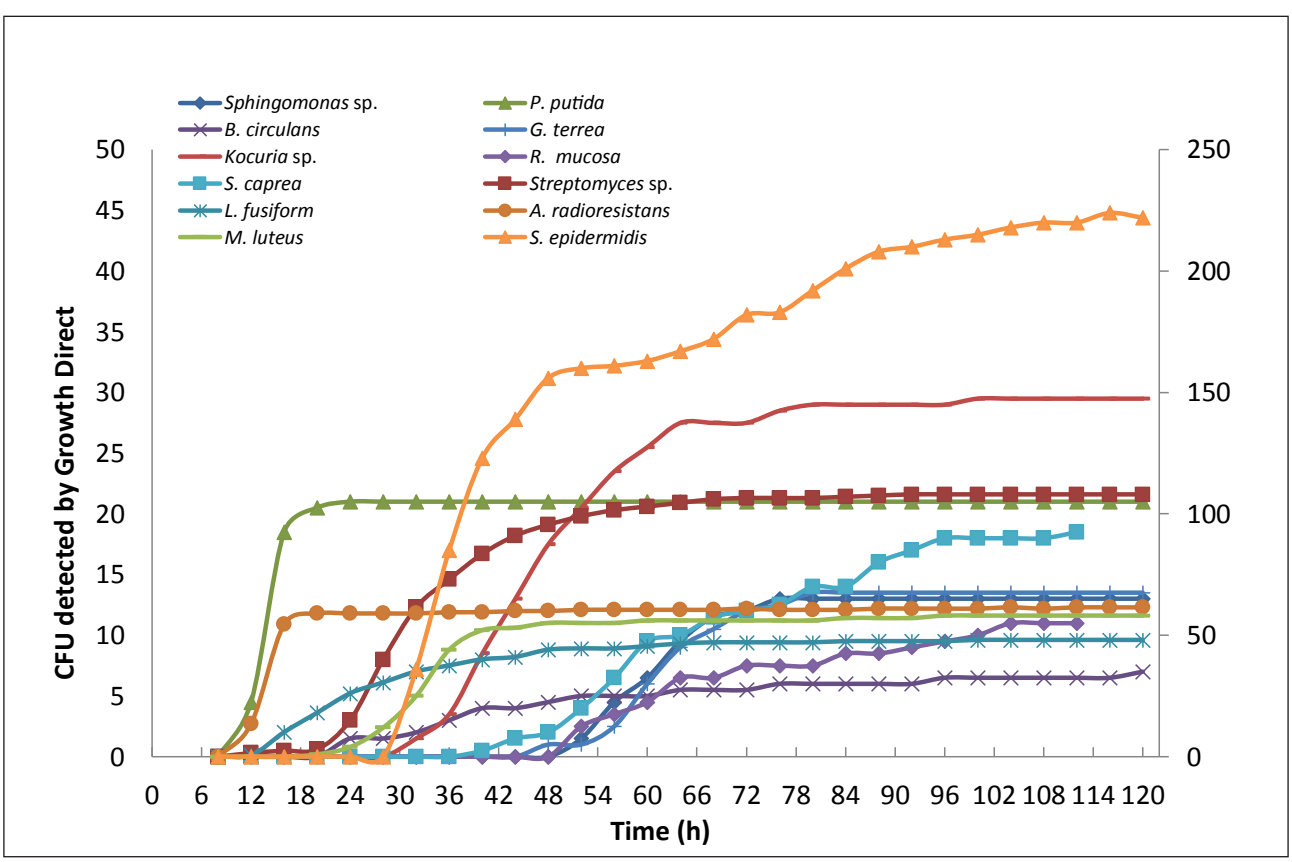

Figure 2a. Bacteria CFU detected at incubation profile of 22.5°C for 5 days.

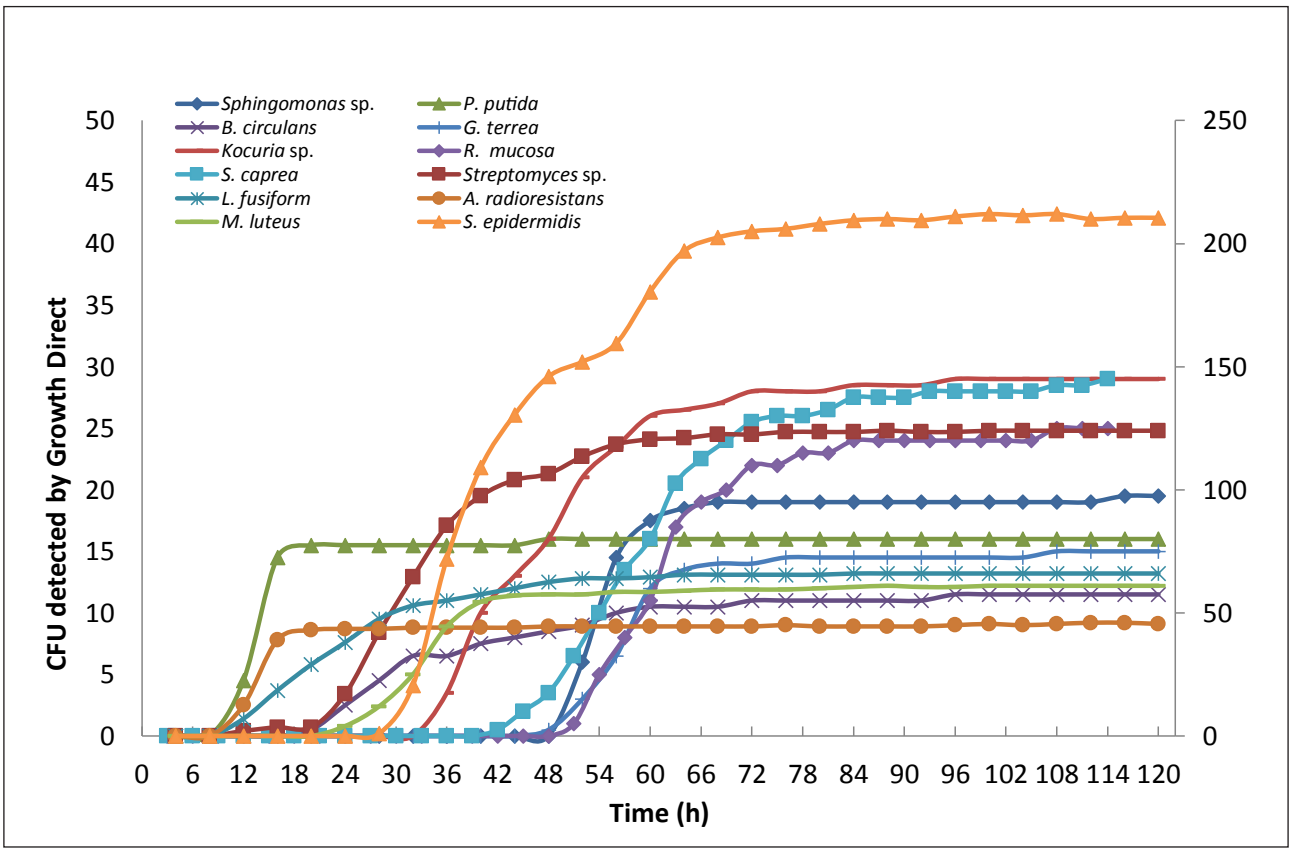

Figure 2b. Bacteria CFU detected at incubation profile of 22.5°C for 2 days and 32.5°C for 3 days.

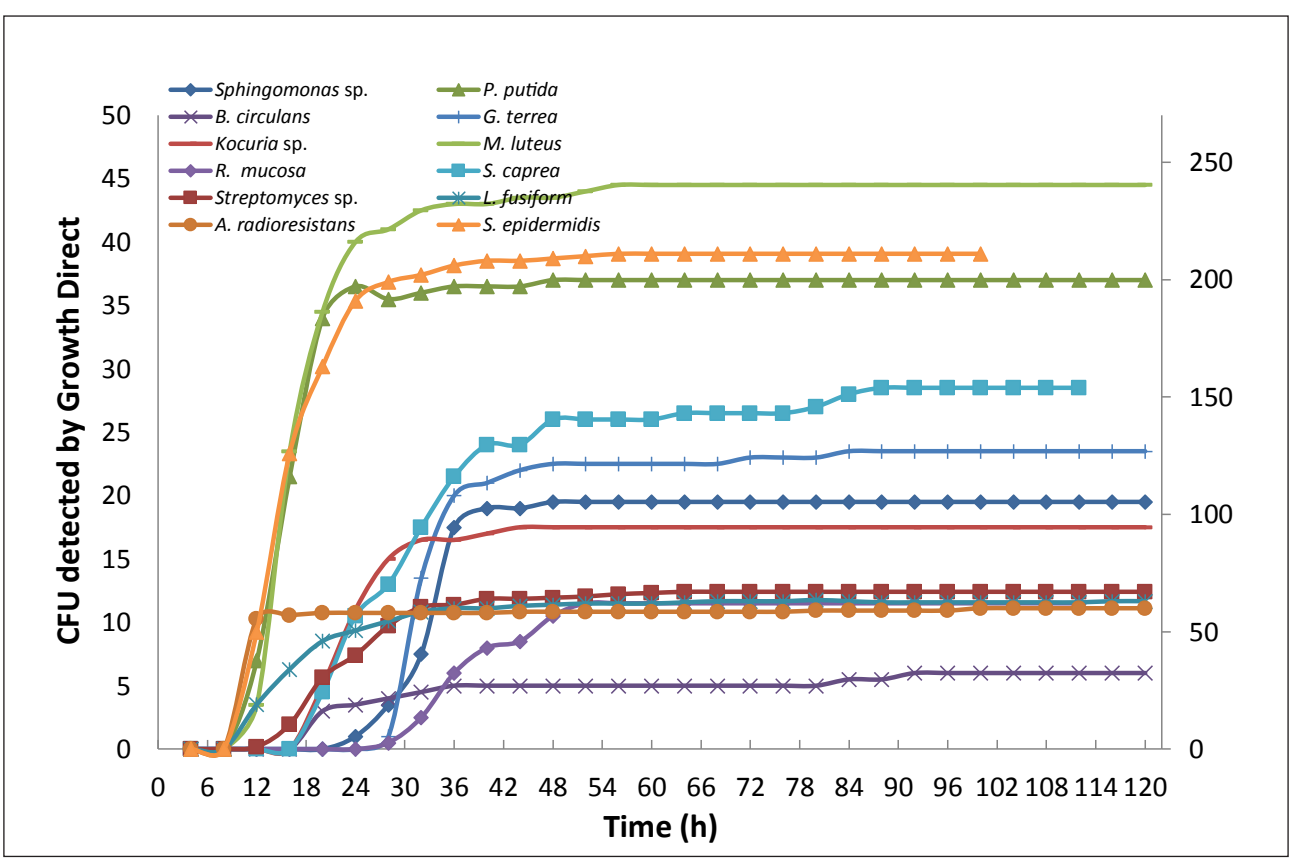

Figure 2c. Bacteria CFU detected at incubation profile of 32.5°C for 5 days.

detection curves of all strains reaching a plateau within approximately 48 h of incubation (**Figure 2c**). The lower 22.5°C incubation profile resulted in slower recovery with CFU detection still not reaching a plateau until after 90 h (**Figure 2a**). The serial incubation resulted in longer detection of bacteria colonies with plateaus in the detection curves obtained in the range of 72 h (**Figure 2b**).

Detection of the three non-temperature-sensitive mould species was variable (**Figure 3**). *Penicillium chrysogenum* and *Erwinia rostatum* exhibited only slightly slower detection at 22.5°C versus 32.5°C (**Figure 3a** and **c**), however, *Aspergillus fumigatus* fully recovered at 22.5°C by the end of the assay as evidenced by the absence of a plateau in its curve, while being detected after 36 h at 32.5°C, and 72 h in the serial incubation (**Figure 3a–c**). The temperature-sensitive strains exhibited limited or no growth at 32.5°C, while exhibiting a plateau in growth by no later than 48 h at 22.5°C.

These results indicate that a single 32.5°C incubation regime would produce the fastest TTR for bacteria, but not for mould species. Temperature-sensitive mould species did not grow at this temperature, and thus would not be detected. A 22.5°C only incubation would result in an unacceptably long TTR that could negate any significant time-saving from the use of the Growth Direct System for testing. Based on these data, the serial incubation was chosen as the most suitable incubation profile both to optimise recovery of the widest range of microorganisms, and obtain the fastest TTR.

TTR determination for the selected serial incubation profile and equivalence testing versus the standard method

To select the TTR for the chosen incubation profile, results presented in **Figures 2b** and **3b** were further analysed. For each test strain, the percentage of CFU detected versus the final count of CFU enumerated by the Growth Direct was calculated and plotted (**Figure 4**). The presumptive TTR was then selected based on the time-point at which 85% of the final count was detected for all the test organisms. The presumptive TTR for this incubation profile was thus determined to be slightly less than 72 h.

Initial confirmation of the presumptive TTR was then performed by comparison of the colonies detected by the Growth Direct System at 72 h with the spread plate controls for each organism after 3 days incubation at 22.5°C and 2 days at 32.5°C. Equivalence was defined as a Growth Direct count at 72 h that was ≥ 70%. **Table 2** presents the results of the analysis. In five cases, organisms on the control plates overgrew and could not be accurately counted by eye. All but one organism exhibited mean counts that were ≥ 70% of the spread plate controls. One species, *Acinetobacter radioresistans*, exhibited 67% recovery versus the control. Also, a number of strains exhibited high recovery compared to the spread controls due to low overall counts (**Table 2**).

Subsequently, equivalence testing was performed on air samples analysed by the Growth Direct System

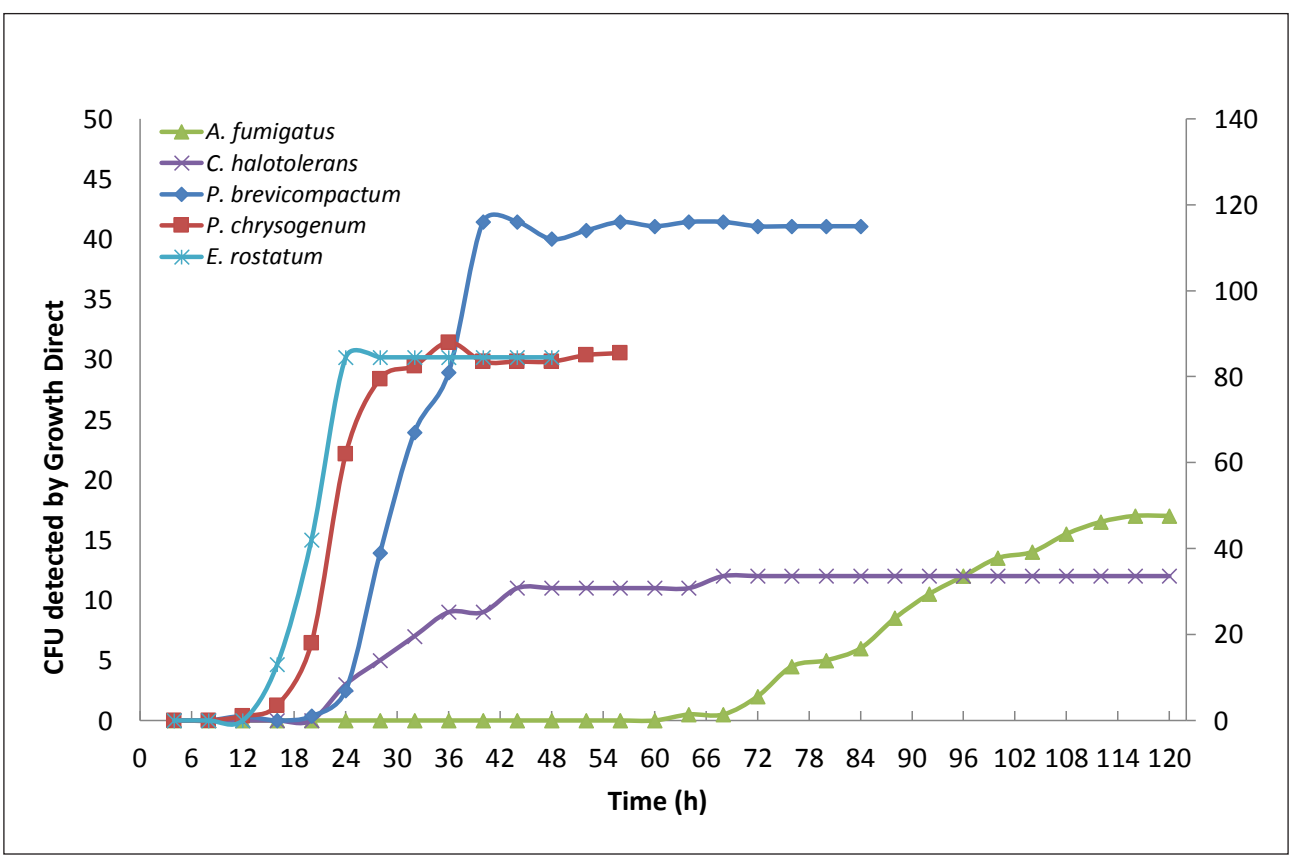

Figure 3a. Mould CFU detected at incubation profile of 22.5°C for 5 days.

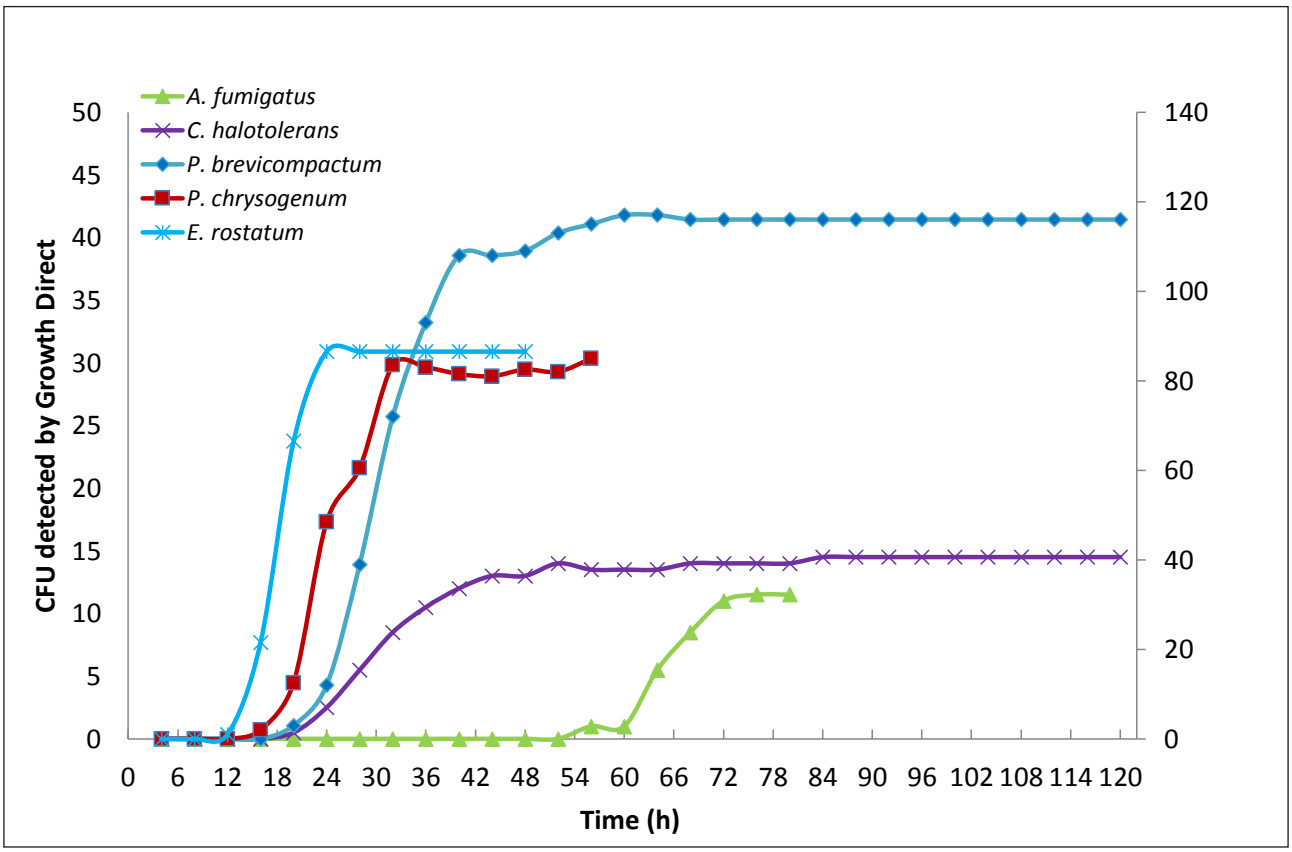

Figure 3b. Mould CFU detected at incubation profile of 22.5°C for 2 days and 32.5°C for 3 days.

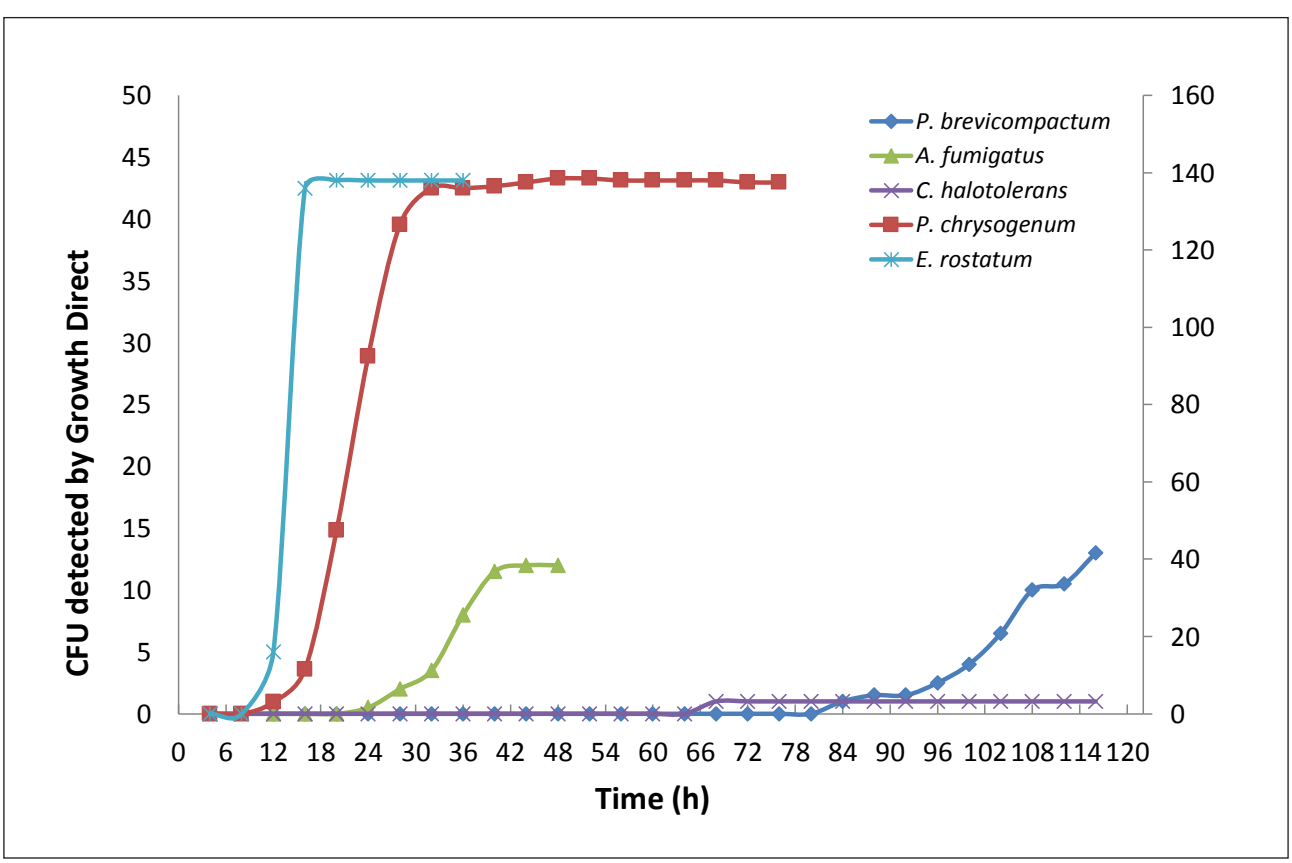

Figure 3c. Mould CFU detected at incubation profile of 32.5°C for 5 days.

Table 2. Comparable recovery of test isolates at 72 h serial incubation versus 5-day spread plate controls.

Organism*	Mean 72 h Growth Direct count (CFU)	Mean control count at 5 days (CFU)	% Recovery
Sphingomonas sp.	19	13	146
P. putida	16	23	70
B. circulans	10.5	5	210
A. radioresistans	44.5	66.5	67
G. terrae	14	16	88
Kocuria sp.	28	26	108
M. luteus	59.5	50.5	118
S. epidermidis	205	137	150
A. fumigatus	11	14.5	76
C. halotolerans	49.5	51.5	96
R. mucosa	22	10.5	210
S. caprea	25.5	13	196
P. brevicompactum	44.5	34	131

* *L. fusiform, Streptomyces* sp., *E. rostatum*, and *P. chrysogenum* exhibited overgrowth of colonies on the control.

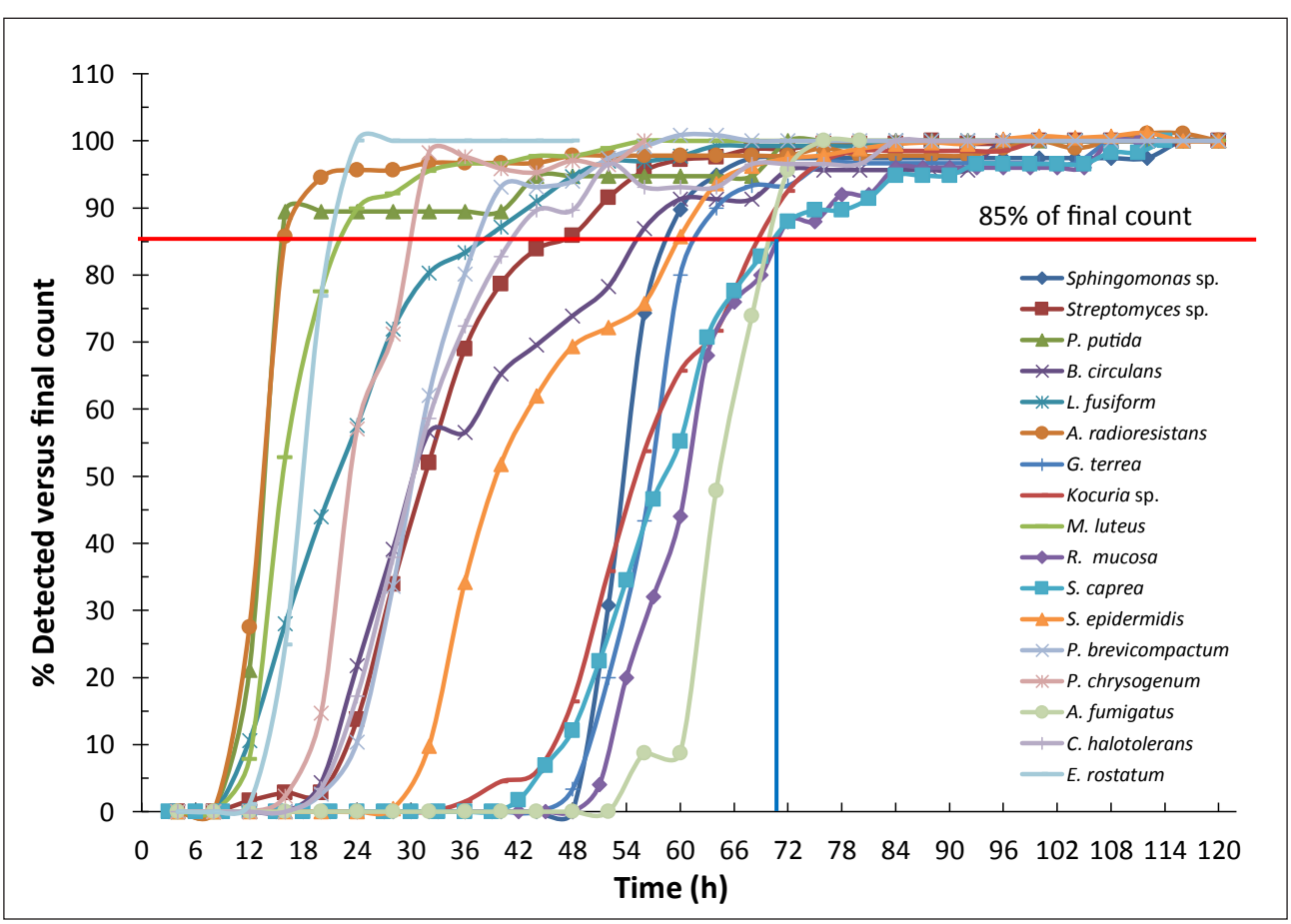

Figure 4. TTR determination at selected serial incubation profile.

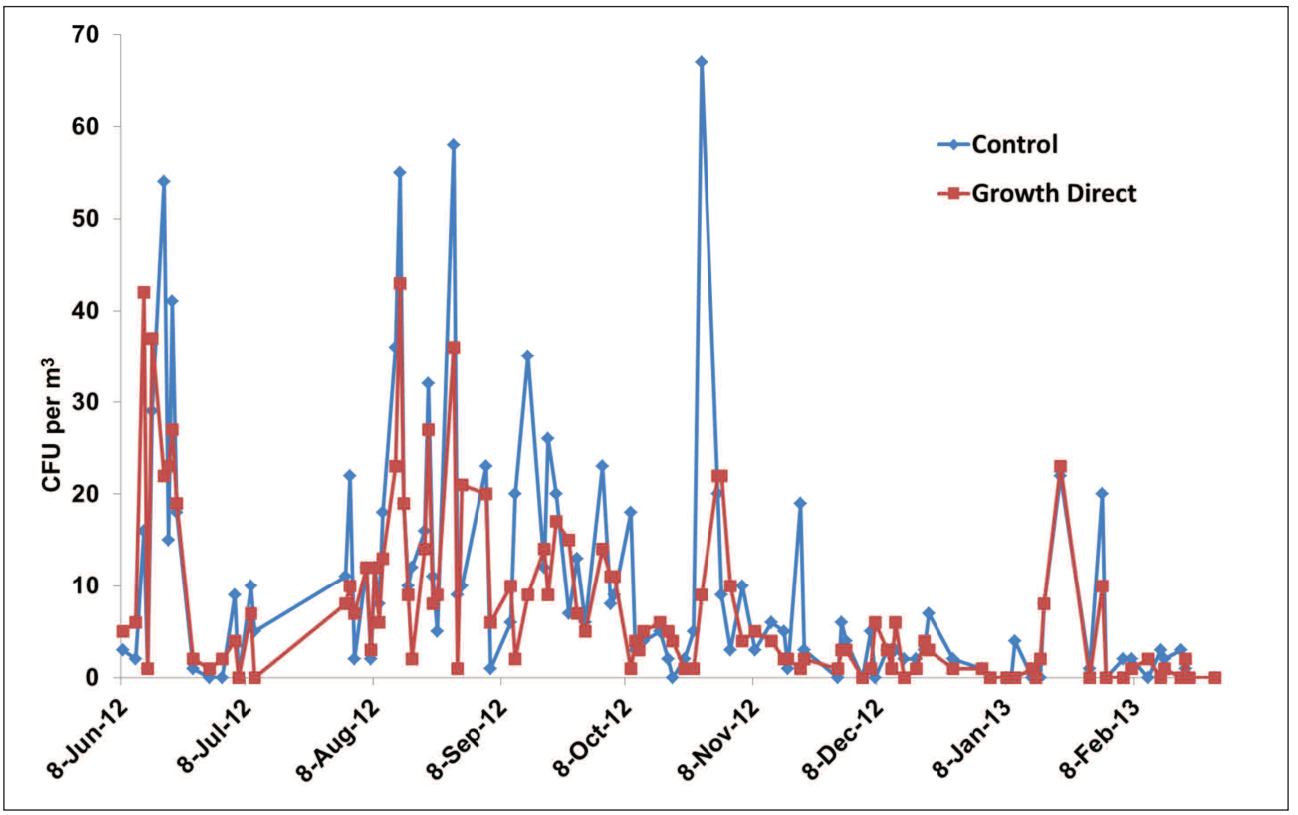

Figure 5. Equivalence testing of Growth Direct System at selected TTR versus standard control method on EM samples.

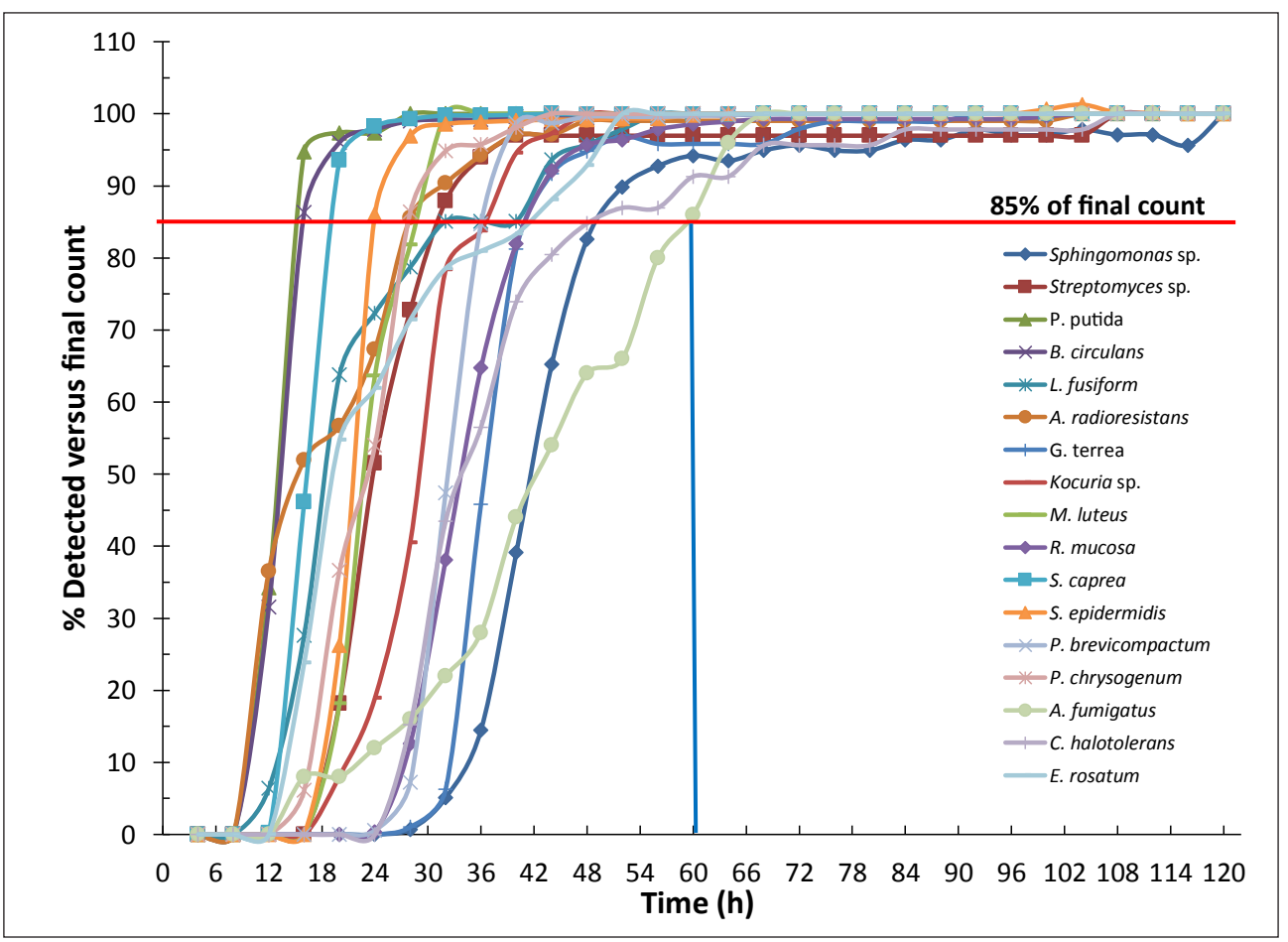

Figure 6. Use of 28°C reduces TTR, while allowing recovery of all strains.

Table 3. Comparable recovery of test isolates at 60 h 28°C incubation versus 5-day spread plate control.

Organism*	Mean 60 h Growth Direct count (CFU)	Mean control count (CFU) at 5 days	% Recovery
Sphingomonas sp.	65	43	151
Streptomyces sp.	16	19.5	82
P. putida	19	20	95
B. circulans	251.5	256.5	98
A. radioresistans	51.5	45	114
G. terrae	46.5	52.5	89
Kocuria sp.	18.5	19	97
M. luteus	42.5	36.5	116
S. epidermidis	311.5	333.5	93
A. fumigatus	21.5	17	126
C. halotolerans	21	27	78
R. mucosa	136	77.5	175
S. caprea	420	437	96

* *P. chrysogenum, L. fusiform, E. rostatum* and *P. brevicompactum* exhibited overgrowth of colonies on the control..

incubated at the selected incubation profile for 72 h versus the standard serial incubation of 22.5°C for 3 days followed by 32.5°C for 2 days. Air testing was performed as it provided more uniform sample capture compared to surfaces. Samples were taken side-by-side using active air impaction systems and trended over a period of several months. The results of the comparison are presented in **Figure 5**. As is shown, the Growth Direct System and standard method counts trended at comparable levels over the course of the study. These data thus demonstrate that the selected TTR using the Growth Direct System resulted in comparable results during normal air bioburden testing.

Alternative incubation strategy to replace serial incubation

Subsequently, the possibility of substituting a single incubation protocol for the serial incubation was examined. The incubation temperature chosen was the mid-point between 32.5°C and 22.5°C at 28°C. This temperature has been investigated as an alternative to serial incubation as it is thought to be low enough to allow the recovery of temperature-sensitive species that would not grow at 32.5°C, while the TTR determined would remain comparable to that obtained for serial incubation as described.

The same species were used in this testing inclusive of the temperature-sensitive moulds described, *P. brevicompactum* and *C. halotolerans*. Each was tested by the Growth Direct System at 28°C for 5 days. As before, counts were detected over the course of the incubation and then the percentage of CFU detected for each time-point versus the final count were plotted (**Figure 6**). The temperature-sensitive moulds grew at this temperature successfully as did the other organisms. Based on the detection plot, the TTR for 28°C was determined to be 60 h for the organisms tested. This was lower than that determined for the serial incubation TTR of 72 h. In addition, recovery of the isolates at the determined 60 h TTR was ≥ 70% of the spread controls for these organisms. Only *P. chrysogenum* exhibited < 70% recovery, but this appears to be a result of variability inherent in the low counts obtained (**Table 3**).

Discussion

As part of their implementation, growth-based RMMs require a TTR to be determined and then verified to confirm that the RMM will provide comparable accuracy to the standard method at the chosen TTR. Selection of the TTR for an RMM must thus balance the requirement for accurate detection of microbial bioburden with the need to obtain as fast a result as possible. Too fast a TTR can result in a failure to detect possible contaminants, while a long TTR may negate any benefits that will be realised from the use of an RMM versus standard agar-based incubation. To obtain a TTR, a simple strategy is to measure the CFU detected by the RMM over the course of the incubation and select the time-point at which the counts obtained are comparable to the final count at the end of the assay. This experimental strategy is vastly simplified if the RMM in question is non-destructive and is also able to perform the enumeration of CFU at each time-point automatically.

The case study presented illustrates this simple strategy to determine a TTR using an automated, growth-based RMM, the Growth Direct System. It should be

noted that, while an EM application was chosen, this same strategy can be applied to a TTR determination for any other testing application. This simple process using less than 20 in-house isolates established a presumptive TTR and completed its initial verification in only a few weeks.

As discussed, a protocol for testing was defined that consisted of testing selected in-house isolates using the Growth Direct System at different incubation profiles to confirm which profile was most appropriate. Based on this data, the serial incubation profile was chosen in order to optimise the recovery of temperature-sensitive moulds as well as the other test strains. Subsequent analysis of the recovery data was performed that determined the presumptive TTR of 72 h. The accuracy of the results using the Growth Direct System was then confirmed against spread plate controls for each organism and side-by-side testing of actual samples. Subsequently, use of a 28°C single incubation regime was investigated as a possible alternative to serial incubation. At this temperature, the TTR of the test isolates was reduced to 60 h while exhibiting the same accuracy as the serial incubation regime. This may thus be a viable alternative regime; however, further equivalence testing will need to be performed in order to verify the validity of the incubation profile for this application.

Conflict of interest

The authors are employed by Rapid Micro Biosystems, the manufacturer of the Growth Direct System.

References

1. Parenteral Drug Association. *PDA Technical Report No. 13 (Revised). Fundamentals of an Environmental Monitoring Program*. Bethesda, MD: PDA; 2001.
2. Food and Drug Administration. *Guidance for Industry: Sterile Drug Products Produced by Aseptic Processing – Current Good Manufacturing Practice. Pharmaceutical cGMPs 2004*. Silver Spring, MD: CDER/CBER/ORA, FDA.
3. Food and Drug Administration. *Pharmaceutical cGMPs for the 21st Century. FDA 1-32*. Silver Spring, MD: Department of Health and Human Services, FDA; 2004.
4. Food and Drug Administration. *Validation of Growth-Based Rapid Microbiological Methods for Sterility Testing of Cellular and Gene Therapy Products*. (2008 Draft Guidance). Silver Spring, MD: FDA; 2008.
5. Yvon P. Viability-based technologies: Digital flow cytometry using Chemunex D-Count® and Bactiflow®. In: *Encyclopedia of Rapid Microbiological Methods Volume 2* (Miller M, Ed.). Bethesda, MD: PDA\DHI Publishing; 2005, pp. 357–378.
6. Parenteral Drug Association. *PDA Technical Report No. 33 (Revised 2013). Evaluation, Validation and Implementation of Alternative and Rapid Microbiological Methods*. Bethesda, MD: PDA; 2013.
7. Moldenhauer J. Justification of incubation conditions used for environmental monitoring. *Am Pharm Rev* 2014; **17**: 44–51.
8. London R, Schwedock J, Sage A, Valley H, Meadows J, Waddington M, Straus D. An automated system for rapid non-destructive enumeration of growing microbes *PLoS ONE* 2010; **5**(1): e8609. doi:10.1371/journal.pone.0008609.
9. Benson RC, Meyer RA, Zaruba M, McKhann G. Cellular autofluorescence – is it due to flavins? *J Histochem Cytochem* 1979; **27**(1): 44–48.
10. Chance B, Thorell B. Localization and kinetics of reduced pyridine nucleotide in living cells by microfluorometry. *J Biol Chem* 1959; **234**: 3044–3050.
11. Aubin JE. Autofluorescence of viable cultured mammalian cells. *J Histochem Cytochem* 1979; **27**(1): 36–43.
12. Dalterio R, Nelson W, Britt D, Sperry J, Tanguay J, Suib S. The steady state and decay characteristics of primary fluorescence from live bacteria. *Appl Spectroscopy* 1987; **41**(2): 234–241.
13. Estes C, Duncan A, Wade B, Lloyd C, Ellis W, Powers L. Reagentless detection of microorganisms by intrinsic fluorescence. *Biosens Bioelectron* 2003; **18**(5–6): 511–519.

Published by **Euromed** Communications
Passfield Business Centre, Passfield, Liphook, Hampshire GU30 7SB, England
Tel: +44 (0)1428 752222 Fax: +44 (0)1428 752223 e-mail: publisher@euromedcommunications.com