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Adaptation of ethidium bromide fluorescence assay to monitor activity of efflux pumps in bacterial pure cultures or mixed population from environmental samples

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ABSTRACT

Quick, simple and reliable assays to screen and understand active efflux pumps operative in pure bacterial cultures or mixed population in environmental samples are important. Although assays are available to monitor efflux activities in pure cultures, none has been used or tested directly on natural samples or cells harvested from them. Here, we have adapted an existing fluorescence assay using ethidium bromide (EtBr) to monitor efflux activities connected to resistance/tolerance to antibiotics (tetracycline), heavy metals (chromium), salt (NaCl) and ionic liquid (1-ethyl-3-methylimidazolium chloride) in different Gram positive and negative bacterial pure cultures. Concentration of 0.5 μg ml^{-1} of EtBr was found to be optimum for the assay. For all tested physiologies, 1.2–2.2 fold lower EtBr fluorescence (or higher efflux) was detected as compared to control. In addition, the assay was efficiently used for the first time to establish differences in efflux activities in microbial cells harvested from different natural environments. Similar to pure cultures, sediments with higher salinity (7%) compared to lower (3%) ones, and antibiotic contaminated hospital drain samples compared to adjoining garden soil showed up to 1.7-fold and 1.3-fold higher efflux activities respectively. Thus, EtBr fluorescence assay can be used effectively in known or novel bacterial physiologies that involve efflux activities not only in pure cultures, but also in natural environmental microbial groups.

1. Introduction

Bacterial efflux pumps are defensive machineries that actively extrude toxic substances from the periplasm and/or cytoplasm. Efflux of tetracycline by *Escherichia coli* cells was the first described active pump mediated physiology (Levy and McMurry, 1978). Subsequently, these pumps were found to be utilized for various fundamental cellular functions like maintaining homeostasis of essential metabolites (Eggeling and Sahm, 2003) and resistance/tolerance against antibiotics, heavy metals, and salts (Du et al., 2015; Nies, 2003; Nikaido and Pagès, 2012; Piddock, 2006; Poole, 2007), that help bacteria to survive in extreme environments. Moreover, efflux pumps are now frequently utilized to bioengineer bacterial strains for several biotechnological applications (Fisher et al., 2013; Mingardon et al., 2015; Yang et al., 2015).

Efflux pumps in bacteria belong to five distinct families based on their transmembrane regions, substrate preferences, and energy sources. These are resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), ATP-binding cassette (ABC) superfamily, small multidrug resistance (SMR), and multidrug and toxic compound extrusion (MATE) family (Piddock, 2006; Poole, 2007). RND superfamily is identified only in Gram-negative bacteria, but the rest are present in both groups (Handzlik et al., 2013). ATP hydrolysis is used exclusively by ABC superfamily members to export their substrates, however the other families use proton motive force (Blanco et al., 2016).

For detection and quantification of efflux activities, molecular techniques like quantitative PCR; immunoblotting; radio-labeled and metal-labeled specific substrates; or non-specific fluorescent

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substrates have been used (Blair and Piddock, 2016; Handzlik et al., 2013; Paixao et al., 2009; Sun et al., 2014; Viveiros et al., 2008). While the first three techniques require prior knowledge of specific target gene/protein/substrate, fluorescent dyes like ethidium bromide (EtBr), Hoechst 33342 or Nile red, can be used as substrate in de novo studies (Olmsted III and Kearns, 1977) (Table 1). Assays utilizing these dyes rely on the principle that fluorescent molecules when used at sub-lethal dose enter cell by passive diffusion, but can be removed only by active efflux system. Therefore, an estimation of relative intracellular concentration of the dye at any point of time can be directly correlated to activity of efflux machinery. In fact these dyes, particularly EtBr, emit very weak fluorescence in aqueous solution (or in media/buffer without viable cells) but the intensity increases with increase in its concentration in the cytoplasm/periplasm of viable cells mostly due to interchelating of the dye to cellular components like DNA (Table 1). Thus, cells with low efflux activity will have higher concentrations of intracellular dyes leading to higher fluorescence and vice versa (Blair and Piddock, 2016). Fluorescence-based assays target the activity of AcrAB-TolC, an RND type multidrug resistant pump in Gram-negative bacteria or ABC and MFS family proteins in Gram-positive bacteria (Coldham et al., 2010; Martins et al., 2011; Paixao et al., 2009; Rodrigues et al,. 2011) (Table 1). All these assays are designed for pure cultures and has never been used directly on environmental samples or cells harvested from them.

The current study provides an adaptation of an already existing EtBr fluorescence assay to monitor activity of microbial efflux pumps in several bacterial pure cultures and harvested cells from natural environments. Sediments of saline environments, and hospital drain soil were tested as proof of concept for rapid analysis of environmental samples.

2. Materials and methods

2.1. Bacterial strains and environmental samples

Bacterial strains with well-known active efflux pump physiologies were used to optimize the assay. This includes a Gram negative multidrug resistant (including tetracycline) strain isolated from poultry fecal samples (Banerjee et al., 2018); The second bacterium is a chromium reducing *Bacillus cereus* strain TCL, isolated from coal mines that use active efflux pump to release the heavy metal from cytoplasm (Banerjee et al., 2019). The third one is a moderately halophilic, Gram negative *Halomonas* sp. (optimum 6% NaCl requirement) isolated from Sambhar salt lake, Rajasthan. This strain was isolated in course of a recent study to isolate 1-ethyl-3-methylimidazolium chloride ([Emim]Cl) tolerant microorganisms (manuscript communicated elsewhere). These imidazolium based ionic liquids are presently the most preferred solvents for lignocellulosic biomass pretreatment in biofuel production and other industries (Dadi et al., 2006; Khudyakov et al., 2012). In a recent study microbial ionic liquid tolerance was found to be mediated by an MFS efflux system (Ruegg et al., 2014). In fact, *Halomonas* sp. could also tolerate up to 5.5% [Emim]Cl and active efflux pumps was found to be involved in the process (manuscript communicated elsewhere).

In addition, sediments from saline environments, and soil from antibiotic contaminated hospital drain, both expected to harbor microbial communities with active efflux systems were used. Saline sediments were collected from mangrove forests of Sunderban, West Bengal, India with 2.5% salinity; and those with 3% and 7% salinity from two different locations of Sambhar salt lake, Rajasthan, India. Sambhar lake is the largest inland saline water body in India that remains dry for major part of the year and thus, sediments from different segments vary in their salinity from 2% to 35% (manuscript communicated elsewhere). Antibiotic contaminated drain soils were sampled from two different nearby hospitals and compared to an adjoining garden soil as control.

2.2. Growth conditions

All experiments with bacterial pure culture were performed in heterotrophic complex media, Luria-Bertani (LB) in aerobic shaking condition at 37 °C, except for *Halomonas* sp. where the LB media was supplemented with 6% NaCl (or as stated in the figure), and 0.2% KCl (Schneegurt, 2012); and pH was adjusted to 8.0 instead of 7.0. *Halomonas* sp. has higher pH requirements as the bacterium was isolated from Sambhar salt lake with pH 8–9. In addition to NaCl (4%, 6%, 10% and 14%), efflux activity in *Halomonas* sp. was also tested in sets with [Emim]Cl (1.8%) and optimum NaCl (6%). For *B. cereus* TCL, 100 $\mu g\ ml^{-1}$ Cr(VI) was used. The strain could very efficiently reduce 200 $\mu g\ ml^{-1}$ of Cr(VI) within 16 h with efficient efflux activity (Banerjee et al., 2019). The commonly used concentration of tetracycline, i.e., 20 $\mu g\ ml^{-1}$, was used for efflux assays, and also for growth, and enumerations. For enumeration of tetracycline resistant bacteria, hospital and garden soils were suspended in PBS buffer and appropriate dilutions spread on antibiotic-supplemented LA plates.

2.3. Processing of environmental samples

Microbial cells in soil or sediments were harvested prior to the assay following a standardized protocol (Sar et al., 2018). Briefly, 5 g sample suspended in 15 ml of 50 mM sodium phosphate buffer was vortexed for 5 min and centrifuged at 1000 g for 10 min to collect the supernatant containing viable cells. Pellet fraction was dissolved again in 15 ml buffer and the steps were repeated to harvest the remaining cells (if any). The pooled 30 ml supernatant was passed through autoclaved filter paper, and the filtrate was centrifuged at 5000 g for 10 min. The pellet fraction containing harvested cells was then used for the assay. When processing sediments from Sambhar salt lake and Sunderban mangroves, NaCl at a concentration equivalent to sample salinity (i.e., 2.5% for Sundarban, and 3%, and 7% for Sambhar) was added to maintain cell stability and viability.

2.4. Efflux assay

20 mg (wet weight) cells from either mid-log grown (OD_{600} = 0.6) pure culture(s) or harvested from environmental sample(s) were dissolved in 2 ml of 50 mM sodium phosphate buffer and used. To this different substrates that trigger efflux pumps in the respective bacteria or environmental samples was added, like tetracycline (20 $\mu g\ ml^{-1}$) for poultry fecal isolate and hospital drain samples; NaCl for *Halomonas* sp. (4%, 6%, 10% and 14%) and saline sediments (3%, or 7%); [Emim]Cl (1.8%) for *Halomonas* sp.; and Cr(VI) (100 $\mu g\ ml^{-1}$) for *B. cereus* TCL. Finally, glucose (0.4%) and EtBr (final concentration of 0.5 $\mu g\ ml^{-1}$) were added to be used as the energy source (Iyer and Erwin, 2015) and non-specific substrate respectively by the induced efflux pumps. Fluorescence emission was monitored immediately for 30 min in a fluorescence spectrophotometer (Hitachi F-7100, Japan) with excitation and emissions at 520 nm and 590 nm respectively (Ferrari and Peracchi, 2002; Tokunaga et al., 2004). The optimized EtBr fluorescence assay to monitor efflux activities operative in diverse groups of bacteria either in pure form or in cell harvests from environmental samples is represented in a flowchart (Fig. 1).

Table 1
List of fluorescence-based studies in bacteria to understand active efflux pumps.

Organism	Gram-nature	Target efflux pump family	Fluorescent dye used	Interaction required for fluorescence (molecule)	Objective of the study	Reference
Escherichia coli	- ive	AcrAB-TolC, RND	EtBr	Yes (DNA)	EtBr efflux kinetics by wild type and mutant strains	Paixao et al. (2009)
E. coli	- ive	Non-specific efflux	EtBr	Yes (DNA)	Intrinsic efflux activity using inhibitors	Viveiros et al. (2008)
E. coli	- ive	AcrAB-TolC, RND	EtBr	Yes (DNA)	Role of calcium in multidrug efflux pump	Martins et al. (2011)
Chromohalobacter sp.	- ive	HrdC (TolC homolog)	EtBr	Yes (DNA)	Induction of multidrug efflux pump by salt	Tokunaga et al. (2004)
Staphylococcus aureus	+ ive	NorA, MFS	EtBr	Yes (DNA)	Inhibition of antibiotic efflux by novel inhibitors	Mullin et al. (2004)
Mycobacterium smegmatis	+ ive	LfrA, MFS	EtBr	Yes (DNA)	Drug susceptibility and EtBr efflux of mutant strain	Rodrigues et al. (2011)
S. aureus	+ ive	LmrS, MFS	EtBr	Yes (DNA)	Role of LmrS in antimicrobial efflux	Floyd et al. (2010)
Mycobacterium tuberculosis	+ ive	MDR	EtBr	Yes (DNA)	Understanding efflux mediated drug resistance	Machado et al. (2018)
E. coli, Salmonella enterica serotype Typhimurium	- ive, - ive	TolC, AcrB and AcrF, RND	Hoechst 33342, EtBr	Yes (DNA)	Understanding efflux mediated drug resistance	Coldham et al. (2010)
Acinetobacter baumannii	- ive	AdeABC, RND	Hoechst 33342, EtBr	Yes (DNA)	Understanding efflux mediated drug resistance	Richmond et al. (2013)
S. enterica	- ive	AcrAB-TolC, RND	Hoechst 33342, EtBr, Nile red	Yes (DNA & membrane)	Efflux pump modulators as antibacterial agent	Reens et al. (2018)
E. coli	- ive	AcrAB-TolC, RND	1,2′- dinaphthylamine	Yes (membrane)	Role of phenylalanine residues in transport	Bohnert et al. (2011)
E. coli	- ive	AcrAB-TolC, RND	Nile Red	Yes (membrane)	Effect of different efflux inhibitors	Bohnert et al. (2010)
E. coli, S. enterica serotype Typhimurium	- ive, - ive	AcrAB-TolC, RND	Doxorubicin	No	Understanding efflux mediated drug resistance using mutants	Blair et al. (2015)
E. coli	- ive	EmrAB, RND	Doxorubicin	No	Function of multidrug resistance transporters	Nishino and Yamaguchi (2001)
E. coli, *Pseudomonas aeruginosa*, *S. aureus*	- ive, - ive, + ive	Non-specific efflux	Fluoroquinolones	No	Accumulation of quinolones in different bacteria	Piddock et al. (1999)
P. aeruginosa, *S. aureus*	- ive, + ive	Non-specific efflux	Fluoroquinolone, radio-labeled	No	Method comparision for measurement of quinolones accumulation	Mortimer and Piddock (1991)
E. coli	- ive	TolC, RND	Trimethoprim (TMP) probe	No	Understanding efflux mediated drug resistance using mutant *E. coli* (Δ*tolC*)	Phetsang et al. (2016)
E. coli (proteoliposomes)	- ive	AcrAB, RND	1-amino-naphthalene-3,6, 8 trisulfonate	No	Function of multidrug efflux	Zgurskaya and Nikaido (1999)
P. aeruginosa	- ive	MexAB-OprM	Nile Red	Yes (membrane)	Effect of glucose in efflux	Iyer and Erwin (2015)
Enterobacter aerogenes	- ive	Non-specific efflux	1,2′- dinaphthylamine	Yes (membrane)	Inhibition of antibiotic efflux by inhibitors	Brunel et al. (2013)

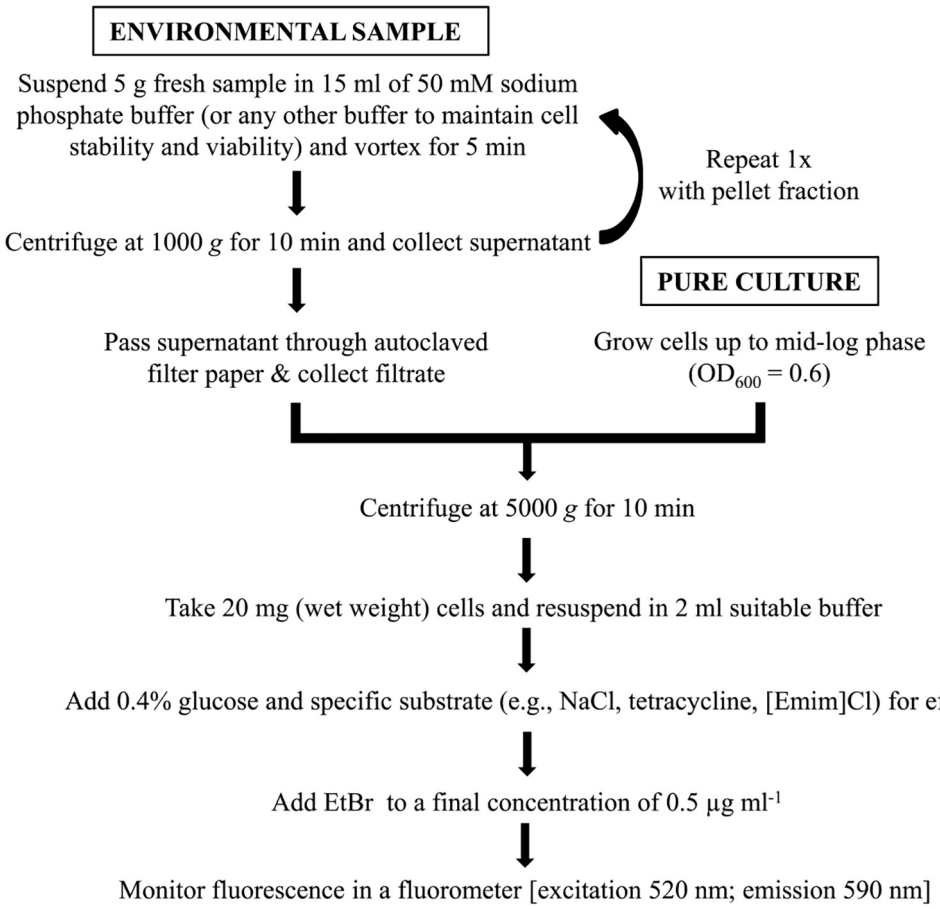

Fig. 1. Flowchart presenting steps involved in the EtBr fluorescence assay to monitor activity of efflux pumps in bacterial pure cultures or mixed population from environmental samples.

3. Results and discussion

3.1. EtBr fluorescence assay can be used to monitor diverse efflux activities in bacteria

Fluorescence assay using EtBr is a well-established method to monitor efflux activities in bacteria. The assay was used to establish its efficiency in different efflux pump mediated microbial physiologies like, tetracycline resistance in a Gram-negative isolate from poultry fecal sample; chromium resistance in *B. cereus* TCL, a Gram positive bacterium from coal mines; and salt and ionic liquid tolerance in *Halomonas* sp., a Gram-negative, moderate halophile. Their minimum inhibitory concentrations (MIC) (or the lowest concentration with no growth) towards the non-specific fluorescent substrate, i.e., EtBr, is 25, 20 and 9 μg ml^{-1} respectively. Thus, any concentration of EtBr that is less than half of these values could be used in the respective strains. However, we need to determine the minimum concentration that would work without losing any signal since we want to exploit the assay directly on environmental samples, where MIC determination is not possible. Thus, we checked 0.25, 0.5 and 1.0 μg ml^{-1} EtBr concentrations in assays with *Halomonas* sp., which have the lowest MIC. While 0.5 and 1.0 μg ml^{-1} EtBr resulted in similar fluorescence, use of lower concentrations (0.25 μg ml^{-1}) leads to loss of signal even in control set. Thus, 0.5 μg ml^{-1} was selected and used for all assays with pure cultures and environmental samples. Similar loss of fluorescence signal was reported with lower EtBr concentrations (<0.50 μg ml^{-1}) in a transporter mutant *E. coli* K12 strain (Paixao et al., 2009).

Involvement of active efflux pumps in all four tested physiologies, namely, resistance/tolerance to tetracycline (20 μg ml^{-1}), Cr (VI) (100 μg ml^{-1}), NaCl (low: 4%, 6%; high: 10%, 14%), and [Emim]Cl (1.8%) in different pure cultures was established by a 1.2, 1.4, 2.2, and 1.7-fold lower fluorescence (i.e., higher efflux activity) in the respective treated sets compared to the controls (Fig. 2a–d). Among the three strains, efflux activities in *Halomonas* sp. was higher for both salt and [Emim]Cl tolerance. Active efflux pumps have previously been reported to be associated with tetracycline (Speer et al., 1992) and chromium resistance (Banerjee et al., 2019; Elangovan et al., 2006; Mishra et al., 2012). Even such pumps are reported to be induced by NaCl (Tokunaga et al., 2004). But, effective use of the assay to detect involvement of efflux pumps in [Emim]Cl tolerance suggests that the assay can be used for other unexplored microbial physiologies that involve active efflux pumps. Or in other words, the assay can also be extended to see if a microbial physiology involves active efflux pump or not.

3.2. EtBr fluorescence assay is effective to monitor efflux activities in environmental samples

Natural sediments from saline environments, and antibiotic exposed soils were used to validate efficacy of the assay. However, natural samples when used directly for the assay did not yield any

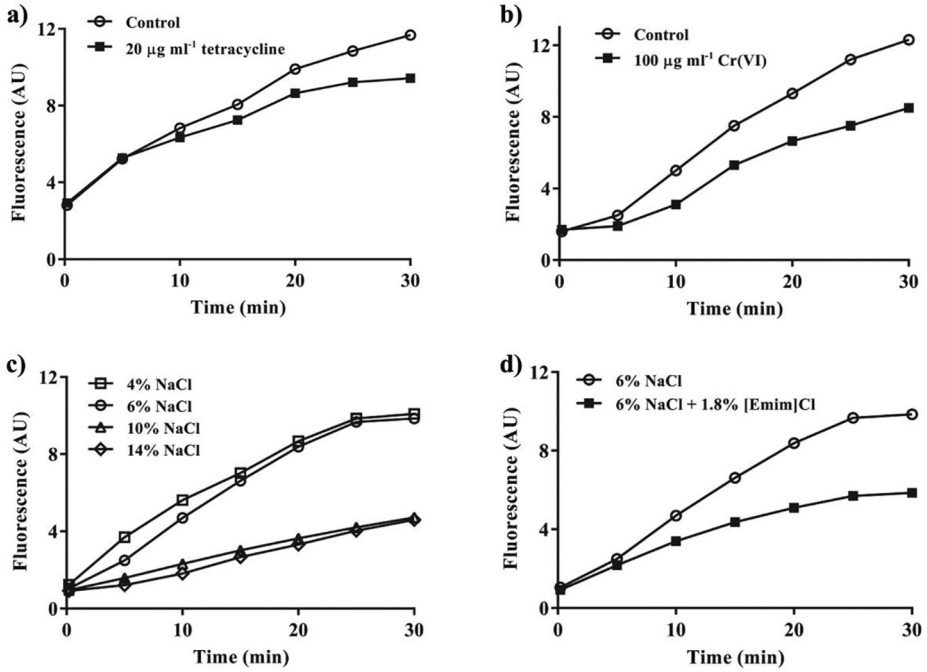

Fig. 2. EtBr efflux assay to monitor active efflux pumps operative in bacterial pure cultures. Fluorescence emission from cells of a) a Gram negative multidrug resistant strain in 0 (control) or 20 μg ml^{-1} tetracycline; b) *B. cereus* TCL grown in 0 (control) or 100 μg ml^{-1} Cr(VI); c) *Halomonas* sp. in 4%, 6%, 10%, and 14% NaCl; and d) *Halomonas* sp. in 6% NaCl (optimum requirement, control) or 6% NaCl plus 1.8% 1-ethyl-3-methylimidazolium chloride, [Emim]Cl. AU, arbitrary unit. Graphs are representative of three independent time scan measurements.

fluorescence, quite presumably due to some inhibitors/interferences present therein. Nonetheless, incorporation of a simple cell harvest step yielded the desired results. The harvest protocol relies on the fact that viable cells would remain in solution and has been previously shown to retain up to 94% of the original cell number in different soil samples (Sar et al., 2018). Cells harvested from Sambhar salt lake sediments with 3% salinity produced 1.7-fold higher fluorescence (low efflux) compared to those with 7% salinity (Fig. 3). However, those from sediments of Sunderban mangroves with only 2.5% salinity showed efflux activities similar to the higher (7%) salinity samples of the salt lake. The possible reason might be the higher nutrient availability in mangrove sediments that supports growth of diverse groups of microorganisms with varying efflux activities.

Similarly, antibiotic exposure in hospital drains is supposed to trigger efflux activities in the residing microbial population. In fact, drain soils from two different hospitals have 1.2 to 1.3-fold lower emission of EtBr fluorescence (i.e., higher activity) than those from a nearby garden soil (Fig. 4a). The observed higher efflux activities in the two hospital drain soils was supported by their higher proportions (6.9% and 7.2%) of tetracycline resistant colonies than the garden soil (0.6%) (Fig. 4b).

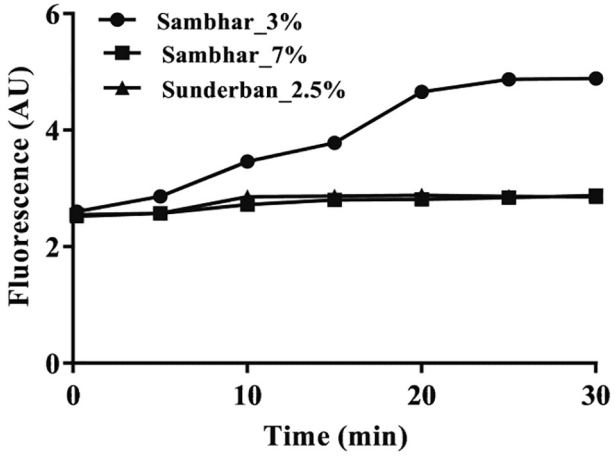

Fig. 3. Fluorescence emission from harvested microbial cells of saline sediments of Sambhar lake (3%, and 7% salinity) and Sunderban (2.5% salinity). AU, arbitrary unit. Graphs are representative of three independent time scan measurements.

4. Conclusion

Thus, EtBr fluorescence assay is a quick, reliable and cost-effective method and its spectrum of use was extended to both Gram positive and negative bacterial pure cultures for known or even new efflux mediated physiologies like ionic liquid tolerance. In addition, the assay was demonstrated to be effective in bacterial pure culture as well as mixed microbial populations directly harvested from natural soil/sediment/water samples. And the same holds true for at least two major efflux mediated activities tested, namely salt tolerance and antibiotic resistance. However, it must also be mentioned that the assay cannot be used as an absolute quantification of the tested efflux activity by all microorganisms present in an environment. Rather, it can be used as an efficient tool to compare such activities in different natural sites. Inclusion of efflux pump inhibitors in the assay will further confirm the process as reported earlier (Viveiros et al., 2008; Bohnert et al., 2010; Brunel et al., 2013). In addition, as natural samples contain different types of microorganisms with varying sensitivities towards EtBr, a very low concentration of the dye must be used that would not become toxic to most microbial groups. Thus, this simple assay can be efficiently used for future explorations of microbial efflux mediated activities in unexplored bacterial cultures and natural environmental samples.

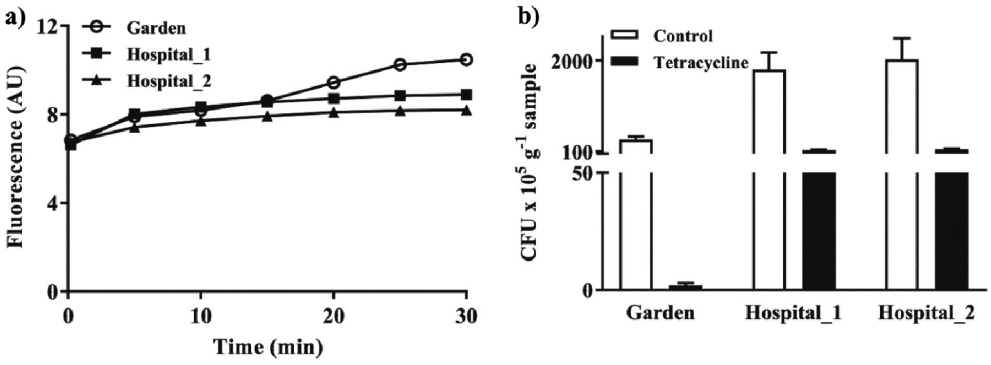

Fig. 4. a) Fluorescence emission by microbial cells harvested from antibiotic-exposed drain soil from two hospitals as compared to those from a non-exposed adjoining garden soil. AU, arbitrary unit. Graphs are representative of three independent time scan measurements. b) Microbial load in the three soils on heterotrophic media and one supplemented with tetracycline (20 μg ml^{-1}). Error bars represent standard deviation of three independent replicates.

Declaration of Competing Interest

The authors declare no conflict of interest.

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