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University of Stavanger

EVALUATION OF DAPI DIRECT COUNT, COMPUTER ASSISTED AND PLATE COUNT METHODS

(Master Thesis in Water Science and Technology)

BOGDAN CHIVU

Spring 2010

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List of abbreviations

A1, A2, A3 – Petri dishes replicates

ABS – absorbance at 600nm

AO – Acridine orange

CA – computer assisted

D0.....D6 – 10.....10^{-6} - diluted samples

DAPI - 4',6-diamidino-2-phenylindole

DNA – Deoxyribonucleic acid

DC – Direct count

FAU – Formazin Attenuation Units

IR – infra red

LED – light emitting diode

MC – manual count

NTU - Nephelometric Turbidity Units

NA – nuclear acid

PP – pseudomonas putida

RNA – Ribonucleic acid

SW – sea water

S_D – standard deviation

S_E – standard error

Turb. - Turbidity

TIFF - Tagged Information File Format

TMTC – Too many to count

UV – ultra violet

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Abstract

The feasibility of using automatic counting of bacteria stained with highly specific and sensitive fluorescing DNA stain DAPI, 4',6-diamidino-2-phenylindole, and direct manual counting to enumerate both pure culture of *Pseudomonas putida* overnight culture and sea water enhanced culture, was tested in correlation with plate direct counting, turbidity and absorbance at 600nm, to obtain cross validation. Six diluted samples from overnight pure culture of *Pseudomonas putida* and sea water culture were diluted and stained with DAPI. The images captured with epifluorescence microscope fit out with a digital camera, were counted automatically using Matlab software and manual counted. In addition, the turbidity and absorbance of both diluted samples was measured, as well as enumeration of colonies in diluted samples plated on the specific growth medium. The results indicated that automatic counting method could be employed successfully in the enumeration of bacteria, but with some limitations due to image analysis technique used and due to limitations of DAPI staining. Also the results indicated that absorbance and turbidity cannot be correlated with the number of bacteria in a sample.

Keywords: DAPI direct count, automatic count, computer assisted count, bacteria enumeration, plate count

1. Introduction

Bacteria are found nearly everywhere. Environmental scientists and microbiologists are frequently interested to find out number of microorganisms in water related with a given environment or process. Counting bacteria in aquatic environments can be used to estimate the biomass in a sample related with a certain environment, to understand the roles of microbes in different water systems. Abundance and biomass of bacteria in sea water are the main parameters to understand the roles of heterotrophic microbes in marine ecosystems (Sherr, 2001).

Progress in microbial ecology has often been stated to be methods limited and progress is impeded by a lack of suitable methods, but it can also be used in the sense of a limiting factor, which implies that any increment in methodology stimulates an increment of progress. Like scientists in other fields seeking to understand complex natural systems, microbial ecologists are faced with the daunting task of finding techniques which provide unequivocal and repeatable results. Microbial systems are so densely multidimensional that it is hard to find and apply methods which address individual dimensions independently enough to meet this requirement (Karen G. Porter, 1980)

Evaluation of bacteria abundance has evolved during the years through different stages: (1) counting bacteria based on the ability of a single bacteria to form colonies on the agar plates, (2) enumeration of total number of bacteria using fluorochrome staining of cells and epifluorescence microscopy and (3) enumeration of phylogenetic

categories of bacterial cells, based on use of specific targeted fluorochromes and molecular probes, via epifluorescence microscopy and flow cytometry (Sherr, 2001).

Direct counting procedure using DAPI staining provide numbers of bacteria two or three times higher than plate counting because direct count are including viable, dead, viable but non cultivable and viable but difficult to culture microorganisms. Another widely used stain for direct counting is Acridine orange (AO) who intercalates with nucleic acids, so the bacteria stained with AO appear green, that indicates high amount of RNA or orange, indicating a high amount of DNA. First in was believed that the green and orange colors are related with the ability of the microorganisms, but is not well established yet (Raina Maier, 2009).

Previous reports in which the two counting methods have been compared have yielded equivocal results. In their original description of the DAPI direct count method, Porter and Feig (1980) found no statistical differences in bacterial counts between DAPI and AO staining methods; however, total counts for cells stained with DAPI averaged 16% lower compared to counts of cells stained with AO. Newell et al. (1986) reported that DAPI sometimes yielded lower cell counts than did AO for samples of estuarine water and suggested that this might be generally true for seawater as opposed to freshwater. The lower DAPI cell cannot be explained also the discrepancy between the low abundance of bacteria in marine water revealed by the plate counting method of viable bacteria and DAPI counting, direct and automatic and are still a controversy that is not resolved yet (Sherr, 1993).

Recently specialized stains became available such as LIVE/DEAD BacLight stain from Molecular Probes. This kind of stains can make the difference between proportion of live and dead cells in the sample. (Sherr, 2001).

The most effective way to alleviate biofouling related problems in water systems is to control the accumulation and activity of the microorganisms responsible for the formation of biofilms. To monitor the bacteria, reliable methods are needed to detect and quantify biofouling. Culturing techniques are conventionally used to enumerate biofouling related microorganisms. There are several limitations in these techniques, not only with respect to the methodology but also in the interpretation of the results and especially in the quantification of sessile bacteria. Therefore the highly specific and sensitive fluorescing DNA stain 4´6-diamidino-2-phenylindole (DAPI), used in conjunction with fluorescence microscopy for counting aquatic microorganisms attached to metal coupons, was evaluated as a method to quantify bacteria on submerged surfaces. When compared with standard plate counts this technique yielded higher numbers of microorganisms and it also simplify the counting of microorganisms and reduce time required to obtain results. (Raina Maier, 2009)

In this experiment the main objective is to compare the automatic enumeration technique used to count bacterial cells after DAPI staining, with direct manual counting. Comparing manual and automatic DAPI direct counts of a defined culture of bacteria and a marine enhancement culture, we can trace correlation plots used for evaluation of both techniques (Kommedal, R. pers. com).

2. Background and Theory

2.1. General description

The quantification of active bacteria is important in studies of microbial production and growth rates, organic matter decomposition and for assigning microbial activities to individual organisms. Rapid enumeration of bacterial cells is important for assessing the movement of different micro-organisms and for preventing bacterial colonization and biofilm formation in the drinking water systems. Bacteria and microorganisms present in water may be major contributors to autotrophic and heterotrophic processes in water systems; however, their detection and enumeration are many times difficult. Direct counts of natural microorganisms collected on membrane filters and stained with a fluorescing dye have yielded higher numbers than other techniques and are considered the most reliable methods available. When excited with light at the appropriate wavelengths, bound dyes produce a fluorescent glow which allows cells below the limit of resolution of light microscopy (generally <1pm) to be visualized and distinguished from other particles, although their shapes and structures cannot be seen. Acridine orange (AO) binds specifically with DNA and RNA under controlled conditions and, when excited with light at a wavelength of 436 or 490 nm, the pure DNA-AO complex fluoresces green and the RNA-AO complex red.

Several difficulties are inherent in the use of AO for direct counts. It is difficult to distinguish bacteria from nonliving particles such as clays,

detritus, or colloids. Auto fluorescence of nonliving matter can mask the auto fluorescence of bacteria and this problem is intensified in waters rich in suspended particulate matter. The standard AO method also requires that slides be prepared from samples within 2 weeks of collection.

The plate count method is the conventional way for estimation of bacteria cells. However, plate count method is time consuming because it needs at least 24 hours of incubation and underestimate the number of bacteria.

A quick method for direct counting of viable and not viable cells using DAPI staining was developed for estimation of cells. Using this method, dead and not dead cells are visualized as blue fluorescent cells under UV excitation employing epifluorescence microscopy.

In the present experiment, plate count, direct DAPI count and automatic DAPI count are compared for evaluation.

2.2. Objectives

In this project the goal is to compare automatic enumeration technique after DAPI staining and epifluorescence microscopy with direct manual count of a pure culture of bacteria (*Pseudomonas putida*) and a marine enhancement culture on a non specific growth medium. The absorbance and the turbidity of each dilution will be measured and the results will be plotted against the manual and automatic DAPI stained bacteria, to see if the turbidity and absorbance at 600nm are in the same range with automatic and direct count.

In addition, the results from a enhanced marine water culture dilutions will be compared with the automatic and manual counting results from the pure culture of *Pseudomonas putida* to trace correlation plots used for cross comparison (Kommedal R. pers. com, 2010)

Also in the experiment the manual count results obtained from plating of *Pseudomonas putida* and marine water cultures will be used for cross comparison of the DAPI stain enumeration technique and plating enumeration technique.

2.3. Main methodology

Pure cultures of *Pseudomonas putida*[1] are prepared on non-specific culture media (agar), by incubating for 24 hours at 30^{o}C. The the absorbance at 600 nm is measured to establish late exponential growth (Kommedal R. pers. com, 2010).

A dilution series of culture are prepared (6 dilutions $10^{-}1,10^{-2}$…..10^{-6}), and three replicate samples from each dilution are filtered onto black membrane filters (0.2 µm, Sigma-Aldrich) and DAPI stained according to the method by Sherr and del Giorgio, 2001. Fifteen TIF images (Tagged Information File Format) are acquired from an epi-fluorescence microscope equipped with a 100x low aperture lens and a DAPI compatible UV cube. Images are analysed in Matlab, a custom made software by Ivar Austvoll, UiS (Tor Myrland, 2009) and

[1] The diverse metabolism of *Pseudomonas putida* may be exploited for bioremediation, for example, it is used as a soil inoculant to remedy naphtalene contaminated soils. *Pseudomonas putida* is capable of converting styrene oil into the biodegradable plasic PHA. This may be of use in the effective recycling of Polystyrene foam, otherwise is thought to be not biodegradable. *Pseudomonas putida* has demonstrated potential biocontroll properties, as an effective antagonist of damping off diseases such as Pythium and Fusarium (Thor, 2009).

compared to manual counts. In addition, all dilution cultures are analysed using optical density at 600 nm and turbidity *(ibid).*

Three replicate marine cultures are prepared by incubating seawater at 30°C for 36 hours at 30^{o}C fed an unspecific culture media (Broth). Using the same approach as for the pure cultures, correlation plots of automatically and manually counted samples are constructed *(*Kommedal R. pers. com, 2010*)*.

2.4. Estimating bacteria on Petri dishes

Because of their very small size, counting the number of bacteria in a sample can be difficult. Although direct counts are possible with a microscope, they require a lot of time and expertise. An easier method is to spread bacteria over a nutrient agar plate and count the number of colonies that grow. If the bacteria are spread out enough, each bacterial cell in the original sample should produce a single colony. Usually, bacterial samples must be diluted considerably to obtain reasonable counts.

To determine the number of cells in a bacterial culture one way of doing this is by carrying out serial of dilutions. Since bacterial cell numbers are usually very high in your original sample, plating out the sample undiluted would just lead to the creation of a bacterial lawn, many individual bacteria colonies that are all growing next to or on the top of another one.

Bacterial cell numbers need to be reduced, which is done by repeatedly diluting the amount of bacteria you have in your sample. A small amount of bacteria sample is mixed with a diluent solution and

then successive dilutions are made. A small amount of each of the diluted bacteria samples is then spread onto an agar plate. The numbers of bacteria colonies that grow on each plate are counted. By working backwards using multiplication with the "dilution factor", the number of times that you have diluted the bacteria sample with the diluent solution, you will be able to estimate the number of bacteria in original sample.

This method has some drawbacks, however. Injured bacteria may not always form colonies. Also, since there is no single diluent solution that supports the growth of all types of bacteria, some bacteria may be left out of any given counting procedure.

2.5. DAPI stain

DAPI or 4',6-diamidino-2-phenylindole is a fluorescent stain that binds strongly to DNA. It is used in fluorescence microscopy, being excited with ultraviolet light. When bound to double-stranded DNA its absorption maximum is at 358 nm and its emission maximum is at 461 nm. DAPI will also bind to RNA, though it is not as strongly fluorescent. Its emission shifts to around 500 nm when bound to RNA.

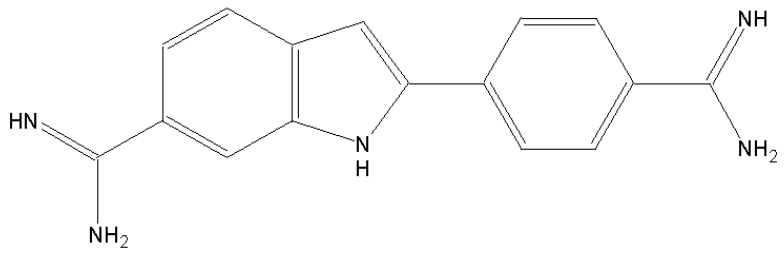
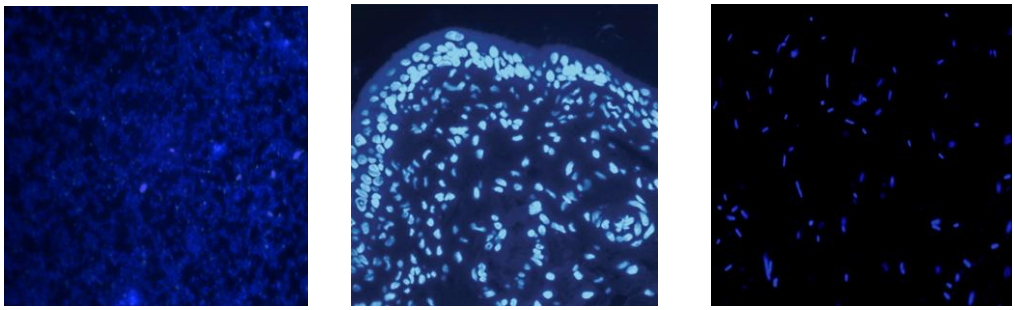


Fig.1 - DAPI- 4',6-diamidino-2-phenylindole – chemical formula

The excitation maximum for DAPI bound to DNA is 358 nm, and the emission maximum is 461 nm. DAPI can be excited with a xenon or mercury-arc lamp or with a UV laser. DAPI may be used in flow cytometry systems utilizing UV excitation sources.



(a) (b) (c)

Fig. 2 - DAPI stained cells of sea water 10^{-1} (a), Pseudomonas *putida* 10^{-1} (b), 10^{-2} (c).

DAPI is a popular nuclear counterstained for use in multicolor fluorescent techniques. Its blue fluorescence stands out in vivid contrast to green, yellow, or red fluorescent probes of other structures. The blue fluorescent DAPI nucleic acid stain preferentially stains DNA but also binds RNA, however in a different binding mode. (DAPI NA Stain, 2006).

Table 1 **-** Properties of DAPI dye (DAPI NA Stain, 2006)

Alternative names	4,6'-diamidino-2-phenylindole, dihydrochloride 4',6-diamidine-2-phenyl indole
Molecular mass	350.25
Excitation wavelength	345 nm (near 360 nm when bound to dsDNA)
Emission wavelength	455 nm (456-460 nm when bound to dsDNA)
Extinction coefficient	~30,000/M cm at 347 nm in methanol

CAS #	28718-90-3
Purity by HPLC	>95% (most lots >98%)
Solubility	Soluble in DMF, water and various non-phosphate aqueous buffers
Storage	Room temperature (RT), protected from light
Reactive groups	Non-covalent; binds to minor groove of double-stranded DNA

The DAPI-RNA complex exhibits a longer wavelength fluorescence emission maximum than the DAPI-DNA complex (500nm versus 460nm) and a quantum yield that is only about 20% as high (*ibid*).

2.6. MATLAB counting software

The name MATLAB stands for matrix laboratory. The MATLAB language is a high-level matrix/array language with control flow statements, functions, data structures, input/output, and object oriented programming features.

To use this program with success it is necessary to organize images and sample directories properly. The program assumes that the images belonging to one sample is placed in a directory with a name like S---. The last four digits after S are used in the name of the file for storing output data. The number of digits used can be changed in the function Count, line 35. All images with extension “TIF” will be processed. If other image formats should be used this can be changed in function dir2imfiles, line 21, (the image is read in function cells).The image directories must be specified as an available path for Matlab. Using “Set path” in the File menu of Matlab can do this (Ivar A., 2010).

The counting is done by dividing the found objects in small, medium

and big objects by their size (area) in number of pixels. The output is given as an estimated total number of cells together with the number of small, medium and big cells. In addition the total coverage in % is also given. To make it possible for further analysis of the results the necessary Matlab data is stored in a separate *.mat file with name given by the sample name and date of the experiment. This mat-file is placed in the same directory as the images and can be loaded from Matlab command window. By the time of finished computation the data will be found in the Matlab structure R placed in the workspace. The data can be opened by the Array Editor (double click on R in the work space list) and studied in detail. Post processing can then be done from the Command window in Matlab (*ibid*).

3. Material and methods

3.1. Counting experiment

The counting experiment consists in following main steps:

1. Starting overnight pure culture of *Pseudomonas putida* at 30^{o} C;
2. Starting three days culture of marine water at 30^{o} C;
3. Preparation of stock culture solutions;
4. Obtaining the diluted samples;
5. Measure the absorbance at 600nm and turbidity for each dilution;
6. Start plating on Petri plates of both pure culture and marine water diluted sample;
7. Count manually the colonies on the plates;

8. Stain with DAPI the dilutions one by one, filter and make the slides for UV microscopy;
9. Capture 15 random pictures from each slide in TIFF format;
10. Count manually the cells in the pictures;
11. Count automatically using Matlab software;
12. Plot the data obtained after counting for comparison.

3.2. The phosphate buffer solution

Phosphate Buffer, near neutral pH, is used in the preparation of dilution blanks for use in microbiological testing. Phosphate Buffer is used rather than unbuffered water in order to standardize this potential variable due to the wide variation in the pH of distilled water from multiple sources.

Preparation of the phosphate buffer used in this experiment: 13,6g KH_2PO_4 was dissolved in 500ml of distilled water at pH 4.5. The solution was bring to pH 7.0 with NaOH 6M. The final solution was bring at a volume of 1l of 0,1 M phosphate buffer around 7.0 pH. .

Because is used in cell culturing, the solution can be dispensed into aliquots and sterilized by autoclaving (20 min at 121°C). Concentrated stock solutions may precipitate when cooled and should be kept at room temperature until precipitate has completely dissolved before use. (Solheim G. 2010)

3.3. Pure culture of *Pseudomonas putida*

Materials needed: Alcohol, sterile nutrient broth solution, agar plates - 3 for each sample, sterile centrifuge tubes, 4 for each sample, a total

of 8, pipettes, 20 glass test tubes for each culture, phosphate buffer 0.1 M, 3 sterile hockey sticks.

After autoclaving the materials which must be sterilized, the plates was prepared and the *Pseudomonas putida* overnight culture and also the sea water culture was started at 30^{o}C in a shaker incubator. The sea water sample must be incubated 36 hours.

3.4. Preparation of stock culture solution

- Transfer 2 x 25 ml overnight culture to sterile nalgene centrifuge tubes, centrifuge at 10000 rpm for ten minutes, decant supernatant and resuspended in 25 ml of phosphate buffer. The operation must be repeated three times.
- Transfer 1ml to first dilution test tube, add 9ml of phosphate buffer and vortex it. This operation must be repeated for all dilutions. The result is 1x10, 1x10^{-1}, 1x10^{-2},…… 1x10^{-6} dilutions.
- Plate out 100 µl of each dilution onto nutrient agar[2] triplicate plates and incubate at 30 degree Celsius.
- Transfer volumes from dilution test tubes and bring volume at least to the 2 ml and add 5% of formalin.

[2] Nutrient agar preparation: suspend 20g in 1l of demineralised water by heating in a boiling water bath or in a current of steam and autoclave for 15 minutes at 121^{o}C. Pour to plates.

- Transfer diluted culture to tubidimeter cuvettes and measure turbidity and also transfers diluted culture to UV-VIS spectrometer cuvettes and measure absorbance at 600nm.

3.5. Counting bacteria colonies on the Petri dishes

Count the number of bacteria colonies that appear on each of the plates that has between 30 and 2000 colonies. Any plate which has more than 2000 colonies is designated as "too many to count" (TMTC). Plates with fewer than 30 colonies do not have enough individuals to be statistically acceptable.

To compute the estimated number of bacteria on the plates, use the following formula:

B = N/D

B = number of bacteria

N = number of colonies counted on a plate

D = dilution factor (either 1, 10 or 100)

Fig. 3 – Plate counting device

3.6. Preparing the DAPI Stock Solution

To make a 5 mg/ml DAPI stock solution, dissolve the contents of one vial (10 mg) in 2 ml of deionized water (dH_2O) or dimethylformamide. The less water-soluble DAPI dihydro chloride may take some time to completely dissolve in water and sonication may be necessary.

For long-term storage the stock solution can be aliquoted and stored at –20°C. For short term storage the solution can be kept at 2–6°C, protected from light. When handled properly, DAPI solutions are stable for at least six months (DAPI Nucleic Acid Stain, 2006).

The excitation maximum for DAPI bound to DNA is 358 nm, and the

emission maximum is 461 nm. DAPI can be excited with a xenon or mercury-arc lamp or with a UV laser. DAPI may be used utilizing UV excitation sources *(ibid)*.

Caution must take when the DAPI solution is handling because is a known mutagen and should be handled with care. The dye must be disposed safely according with applicable regulations *(ibid)*.

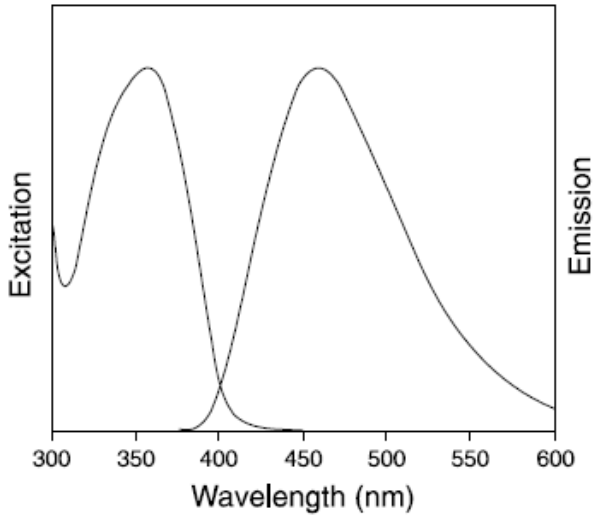


Fig. 4 - Fluorescence excitation and emission profiles of DAPI bound to DNA (After DAPI Nucleic Acid Stain, 2006)

3.7. Filtration and slide preparation

To obtain the slides with DAPI stained samples from dilutions of overnight culture of *Pseudomonas putida* and also from sea water the filtration must be done, using a vacuum filter:

1. Moist cellulose backing filter with some drops of deionized water.
2. Install the polycarbonate membrane in the filter with the glace part up and center it.
3. Put 2ml of sample in a vial using a syringe.
4. Stain with 50µl of DAPI solution using a pipette and let the vial in the dark 10 minutes.
5. Fill the glass tube of the filter with the sample from the vial.
6. Make vacuum and start filtration.
7. With the vacuum still on, kindly remove the backing filter from the cellulose filter with a pincer and install it on the slide, after put a drop a immersion oil on the slide.
8. Cover the backing filter with a glass spangle and remove all the air bubbles using a pincer.
9. Store the slides at -20^{o}C.

(Bagi, Andrea, 2010)

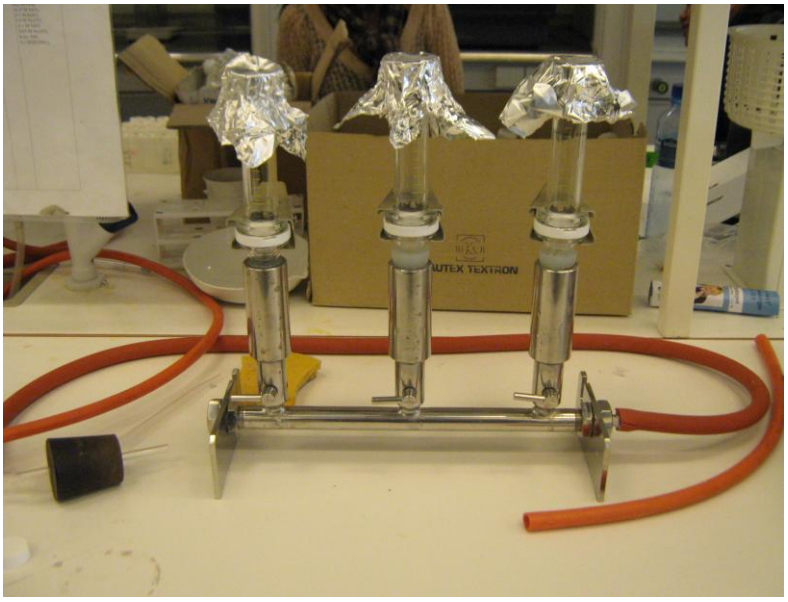


Fig. 5 - Vacuum filter

3.8. Preparation of diluted samples

The procedure to obtain six diluted samples from overnight culture of *Pseudomonas putida* (75ml) incubate in a shaker incubator from 17:00 until 9:30 at 30^{o}C and 120 rpm is presented below:

1. 25ml of overnight culture was pipette in two centrifuge tubes.
2. Two centrifuge tubes were fill-in with 25ml each with phosphate buffer 0,1M.
3. The tubes were centrifuged at 10000 rpm for 10 minutes and decanted the medium from the cells.
4. Added 25 ml of phosphate buffer at 0.1 M in the tubes and resuspended through vortex mixing.
5. The operation was repeated three times.

Observation: The tubes must be put in the same way into the centrifuge each time.

3.9. Shimandzu Uvmini-1240

This spectrophotometer measures absorbance or % transmittance of a sample at fixed wavelength, for example bacterial growth or DNA concentration. Also the device can create a calibration curve from a standard sample and quantifiers an unknown sample.

Spectrum: scans a wavelength range to measure the absorbance and % transmittance of a sample as a function of wavelength. In order to measure spectrum the following settings must be done:

From the “Mode menu” select the mode you want to use:

- Use GOTO WL key to set the wavelength;

- T% ABS key is used to switch between % transmittance and absorbance:
- To do blank correction, set the blank sample in the sample compartment and press the AUTO ZERO key.

To measure spectrum of sample, the steps below must be followed:

1. Measurement mode: ABS
2. λ range: 600 nm – 240 nm
3. Rec. range : -0,5 – 3.0
4. Scan speed: medium
5. No. of scans: 1
6. Display mode: Sequen

This is the mode for setting of the instrument; any change must not be done if the operator not knows better what it means the settings. (Shimandzu, 2010**)**

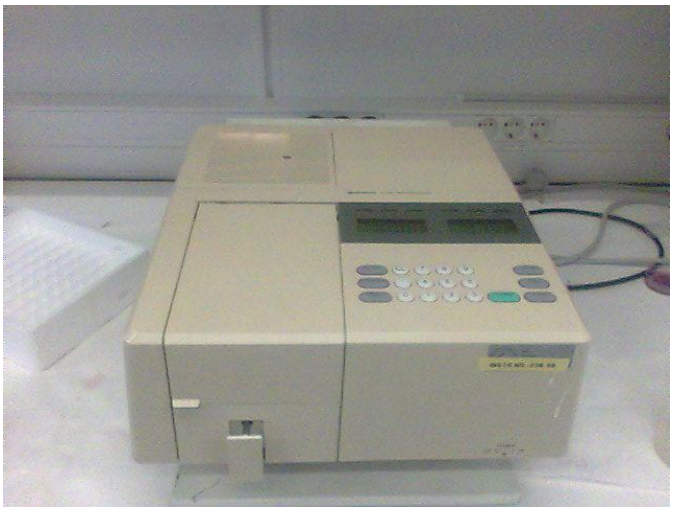


Fig. 6 - Shimandzu Uvmini 1240

3.10. Turbiquant 3000 IR

Turbiquant 3000T/ Turbiquant 3000 IR lets you perform turbidity measurements easily and reliably. The measuring method used in the Turbiquant 3000 IR corresponds to ISO EN 7027 and follows the US EPA construction recommendations. This instrument authorized exclusively for turbidity measurements in the laboratory.
Technical data:

- Depth: 290 mm
- Width: 252 mm
- Height 100 nm
- Weight 1 kg
- Sample temperature: 10-40 gr. C
- Ambient temperature 10-15 gr. C
- Measuring principle: Nephelometric, Ratio method selectable; Transmission Turbiquant 3000 IR only.
- Light source: Turbiquant 3000T- tungsten lamp; Turbiquant
- 3000IR-infrared LED

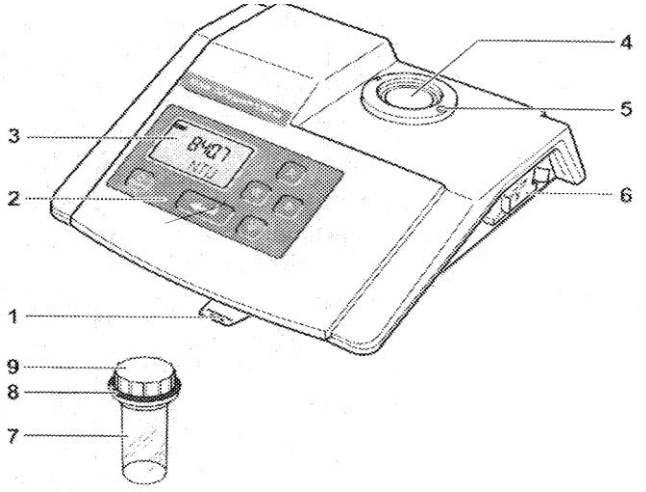


Fig. 7 -Turbiquant 3000 IR

1-sort instructions, 2-keypad, 3-display, 4-cuvette shaft, 5-marker pin, 6-lamp module, 7-cuvette, 8-marker ring, 9-light protection cap.

Operation of instrument

For routine measurements without special accuracy requirements, a warm-up period of 5 minutes is sufficient, but for high precision measurements, the warm-up period should be 30 minutes minimum. This is also valid when you want to calibrate your instrument and only in that way is guarantee that the instrument measures with accuracy specified in technical data. Even the instrument is not measuring, always leave a cuvette in the cuvette shaft so that the cuvette shaft is protected against dust.

The cuvette must be absolutely clean, but even completely clean quality cuvettes exhibit tiny directional differences in their light transmittance. Therefore, each cuvette should be marked, both the measuring cuvette as well as cuvettes with calibration standards. Consequently, each cuvette must be always be inserted in the correct position and you can achieve precise measuring results.

Gas bubbles in the sample affect the measuring result to a massive extent because they have a large scattering effect on the incident light. Larger gas bubbles cause sudden changes measured values whereas smaller gas bubbles are recorded by instrument as turbidity. Therefore, avoid or remove gas bubbles during sampling, if necessary vent the sample (ultrasonic baths, heating or adding a surface tension) or use pour-through assembly (Turbiquant, 2010).

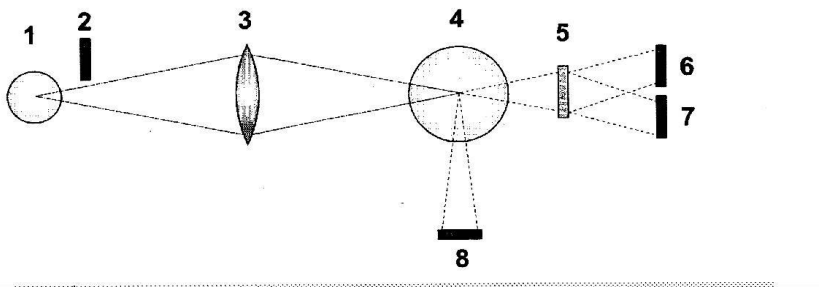


Fig. 8 – Turbiquant 3000 IR measuring system

1-light source, 2 reference detector, 3-lend, 4-cell with sample, 5-beam splitter, 6-transmission detector for low transmission (high turbidity), 7-transmission detector for high transmission (low turbidity), 8-nephelometric detector. *(ibid)*

The measuring system consists of a light source, the cuvette with the sample, a beam splitter and a total of four light detectors.
The instrument can measure with different measuring methods; the signals of the four detectors are evaluated differently:

- Nephelometric (90 degrees scattered light) measurement: the rays of the undisolved particles scattered in a 90 degrees angle are measured. The measuring result is given in NTU.
- Transmission measurements: the intensity of the rays going through the sample is measured. The results are given in FAU

The turbidity of the samples can be measured in this way:

1. Rinse out a clean cuvette with the sample to be measured. Pour approximately 20 ml sample into cuvette. Close the cuvette and rotate it several times before throwing the sample away. Repeat the rinsing procedure twice more.
2. Fill the cuvette with the sample to be measured, aprox. 30ml.

Close the cuvette with the black light protection cap.

3. Make sure that the outside of the cuvette is clean, dry and free of fingerprints.
4. Insert the cuvette in the shaft of the turbidimeter.
5. Align the cuvette and wait for a stabile measured value and read the value.
6. Repeat steps for all dilutions (*ibid*).

3.11. DAPI cell counting

Number of the cells in the sample was monitored by filtering, DAPI staining and microscopy. The analysis method was adapted after Sherr, B et al. (Paul, J. H., 2001). Polycarbonate, 0.22 micron, black filters, 25 mm diameter AP-20 Millipore was used for the filtration, on a filter apparatus. DAPI, 4,6-diamino-2-phenylindole, 50µl / sample was used as stain for bacterial cells (Sherr, 2001).

A Nikon epifluorescence microscope, with a 100/1.3 – oil objective equipped with a digital VisiCam camera with a maximum resolution of 2592 x 1944 pixels using a mercury lamp powered by a power supply Nikon HBO 100W, was used to capture random images from the filter surface. A set of 15 TIFF images at a resolution of 2592 x 1944 was taken for each dilution of bacterial cells and each diluted sample was quantified using a Matlab software for cell counting and manual counted (Kommedal, R., 2010 - personal communication).

3.12. Computer assisted count using Matlab

Before starting the count procedure it may be necessary to inspect the images in the sample folder. In Windows XP this is convenient using the slide show. If some odd image is found which may give unfaithful counts it should be removed from the sample folder.

To use Matlab software to estimate the number of DAPI cells in a picture, the steps below must be done:

- Specify the directory name above all the sample directories:

pathname='C:/directory name';

- Start the computation by calling NewCount. A window will appear where the sample directory must be chosen:

>> R1=NewCount (pathname, [20 700 2500, 3]

The name before the equal sign can be chosen freely.
Another useful post processing function is ViewNImage. This can be used to display an image showing marks of how the counting performed.

>> ViewImage (R2, 1, 1);

The two numbers following the data structure, R6 in this case, is the first image number (here image number 1 for sample 2) and the last figure number (Ivar, 2010).

4. Results and discussions

4.1. Turbidity and absorbance

In the tables below are the values of absorbance at 600nm and the turbidity of each of the six diluted samples from *Pseudomonas putida* overnight culture and sea water enhanced culture.

Table 2 - Absorbance at 600nm

Absorbance at 600nm							
	1x10	$1x10^{-1}$	$1x10^{-2}$	$1x10^{-3}$	$1x10^{-4}$	$1x10^{-5}$	$1x10^{-6}$
Pseudomonas putida	0.784	0.069	0.014	0.008	0.002	0.005	0.003
Sea water	0.785	0.05	0.04	0.016	0.015	0.016	0.014

Table 3 - Turbidity

Turbidity							
Dilutions	1x10	$1x10^{-1}$	$1x10^{-2}$	$1x10^{-3}$	$1x10^{-4}$	$1x10^{-5}$	$1x10^{-6}$
	D0	D1	D2	D3	D4	D5	D6
Pseudomonas putida	59.6	7.44	1.41	0.65	064	0.65	0.56
Sea water	145.14	92.68	25.67	12.63	11.58	4.7	4.1

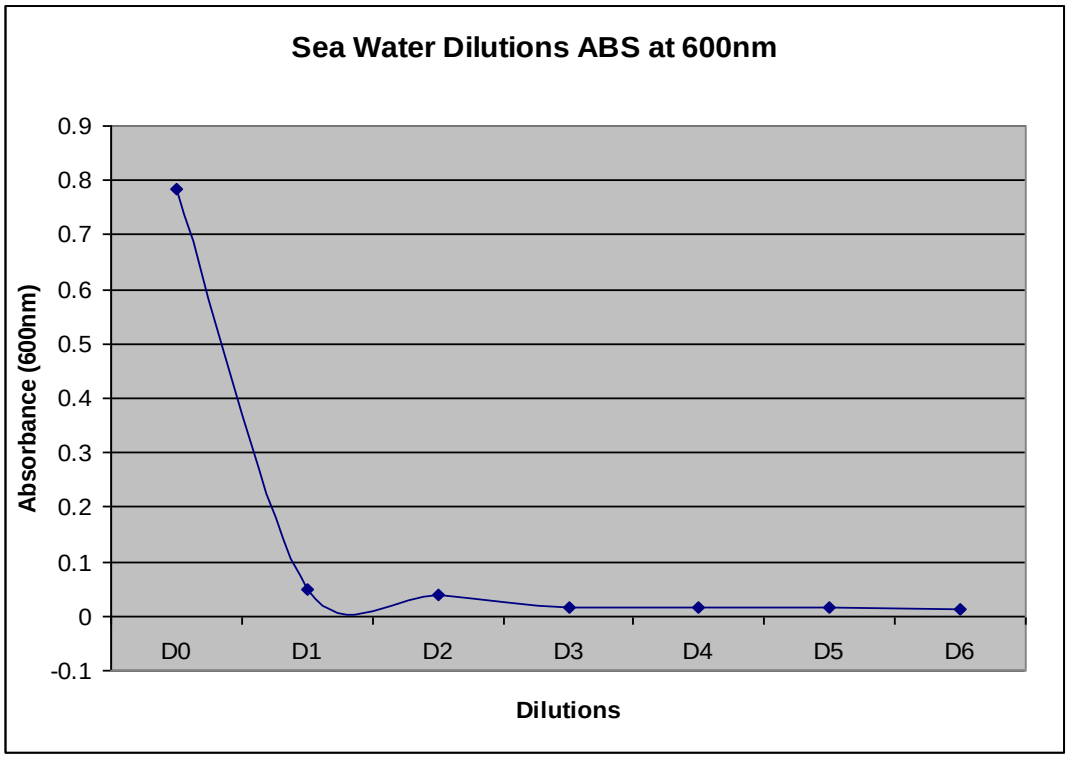


Figure 9 – Sea water dilutions absorbance at 6000nm

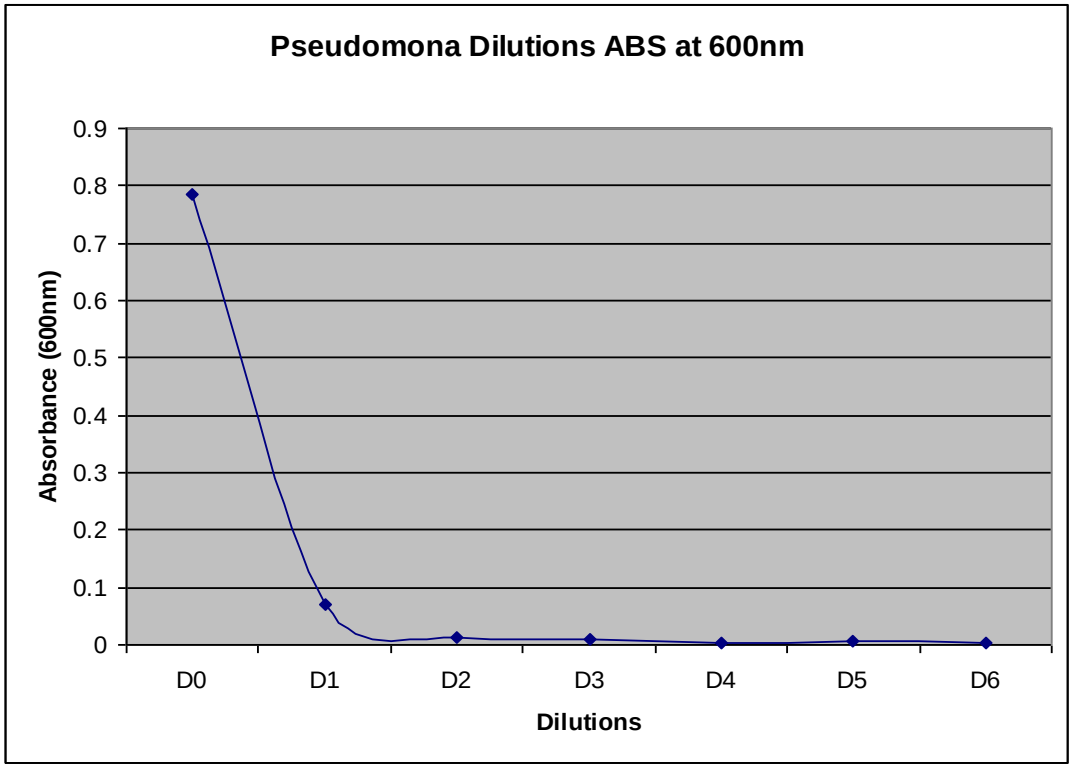


Figure 10 – Pseudomonas dilutions absorbance at 6000nm

It can be observed from figure 9 and 10 that absorbance in both *Pseudomonas putida* pure culture and sea water diluted culture, remain constant from dilution 10^{-2} to 10^{-6} the last dilution. This means that absorbance at 600nm cannot be correlated with the number of cells in the diluted samples because on the low dilutions the results are not relevant.

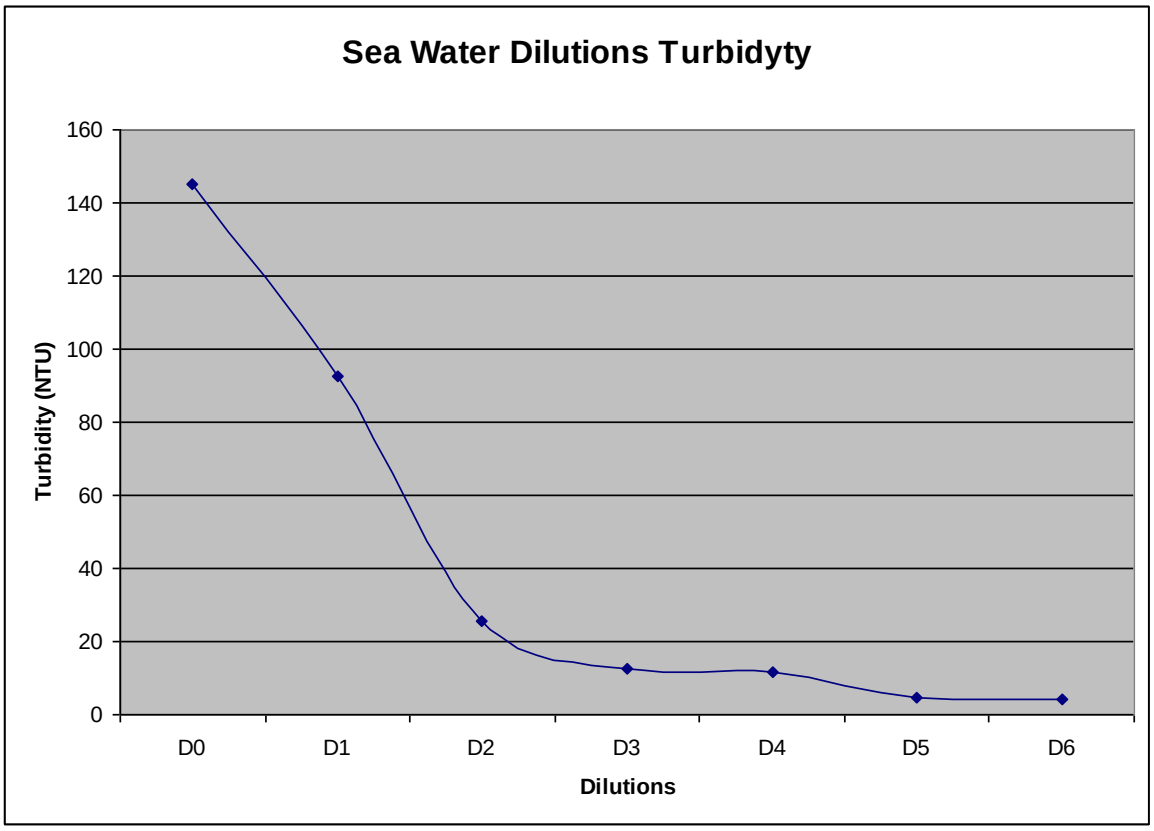


Figure 11 – Sea water dilutions turbidity

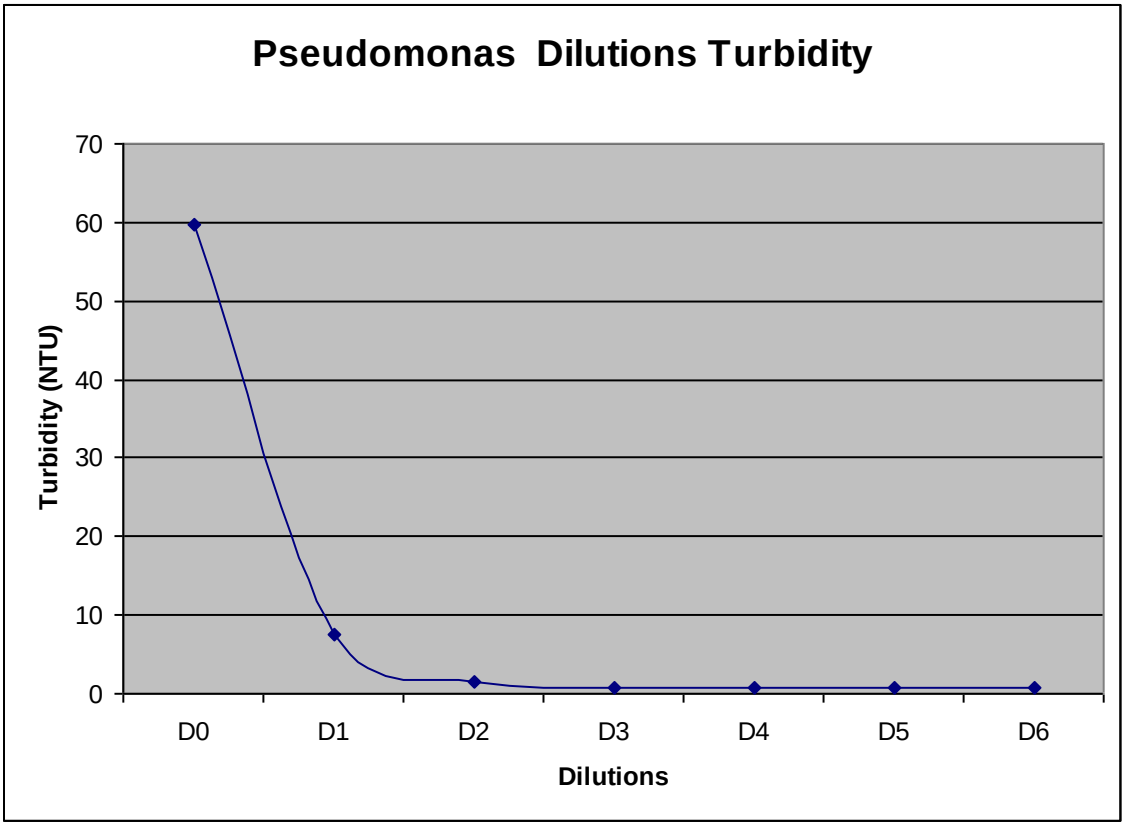


Figure 12 – Pseudomonas dilutions turbidity

In figure 11 the turbidity decreased with dilution, but because the sample is from sea water which contain also different kind of particles and chemicals dissolved, like salts for example. In the case of pure culture of Pseudomonas, the graph is obvious different.

In figure 12 it can be observed that, like in the case of absorbance showed in figure 9 and 10, there are not relevant data between 10^{-2} and 10^{-6} dilutions.

4.2 Computer assisted and manual DAPI count

To perform manual DAPI count, all the pictures was color printed on A4 paper format and every cell or group of cells was marked with a pencil. Some errors occur in manual count because of stained cells stacked together. In this case only a evaluation of the number of cells in the stacked cells group was possible. Anyway, the Matlab software also estimates this group of stick cells, thus the results can be compared successfully.

Table 6 - Pseudomonas putida dilutions DAPI manual count

Pseudomonas putida manual count						
No. of picture	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
1	253	61	22	2	3	1
2	412	69	15	5	2	4
3	324	48	16	4	9	1
4	356	43	31	5	7	2
5	278	50	25	7	0	2
6	370	96	13	5	1	0
7	426	68	18	6	6	0
8	465	112	15	2	2	0
9	315	89	17	8	0	3
10	264	93	18	2	4	0
11	480	70	21	2	8	0
12	401	60	18	2	3	0
13	372	96	34	1	0	1
14	282	97	11	11	11	2
15	363	101	19	1	3	2

Table 7 – Sea water dilutions DAPI manual count

Sea water dilutions manual count						
No. of picture	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
1	1032	387	82	59	2	1
2	625	402	212	49	3	2
3	787	314	145	39	5	5
4	787	267	210	12	4	5
5	104	299	267	19	4	7
6	1734	311	134	11	3	2
7	1348	324	156	7	5	3
8	1306	286	151	8	11	4
9	1178	322	53	15	9	10
10	844	245	76	18	9	3
11	380	324	38	13	12	1
12	1167	356	52	9	1	4
13	1098	327	149	14	3	2
14	1392	336	37	16	0	0
15	245	312	74	15	0	0
Average	935	320	122	20	5	3

The results in the tables 6 and 7 are given in number of cells/picture.

One picture has 0,216mm^2

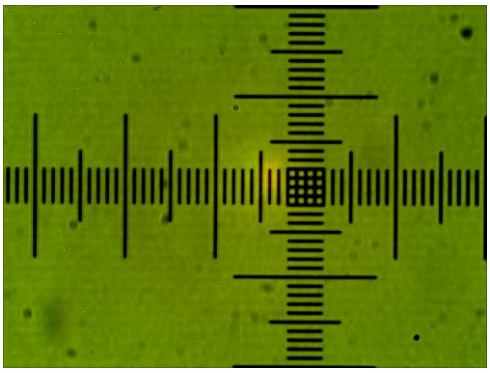
Aria of the filter: 314mm^2

Amount of diluted sample: 2ml

No. of cells / µl = 14,537 x no. of cells in the picture

Example: picture D2-1 has 387 x 14,537 cells / µl

D2-1 = 5625, 8 cells / µl



Calibration slide: 1Div.= 0,01mm

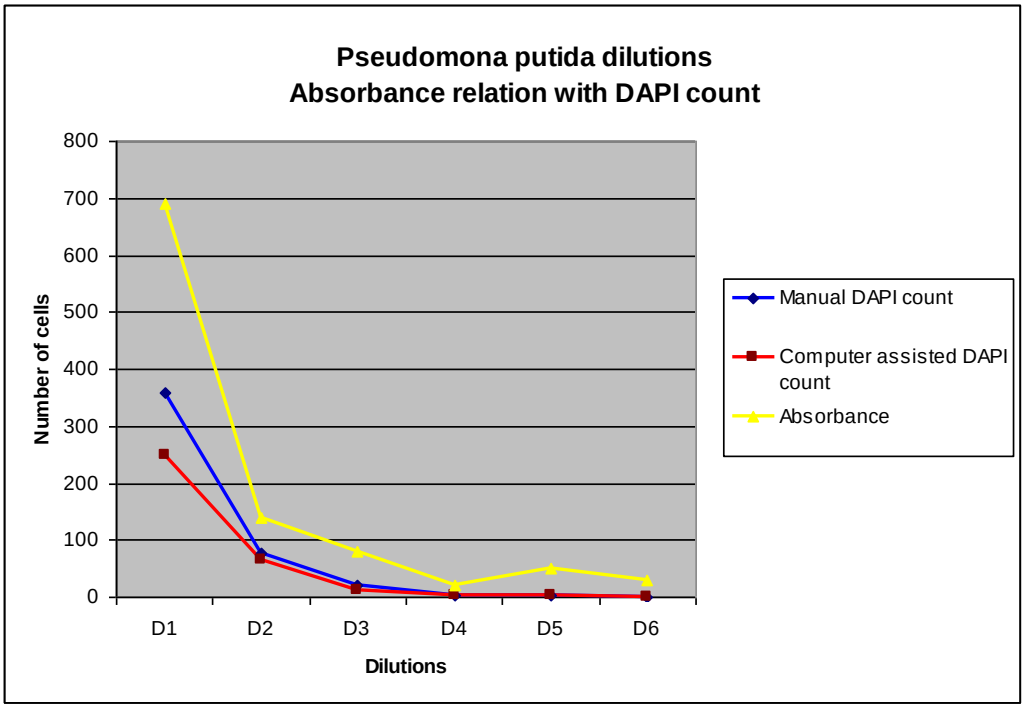


Figure 13 –Comparison between DAPI count and ABS in *Pseudomonas dilutions*

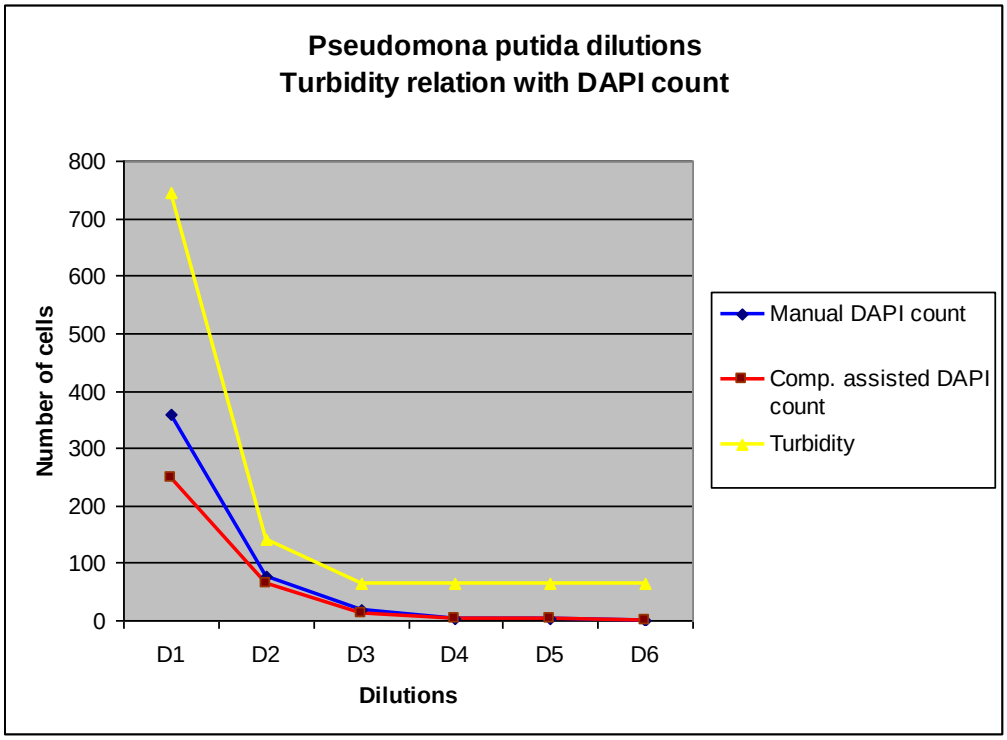


Figure 14 –Comparison DAPI count and turbidity in *Pseudomonas dilutions*

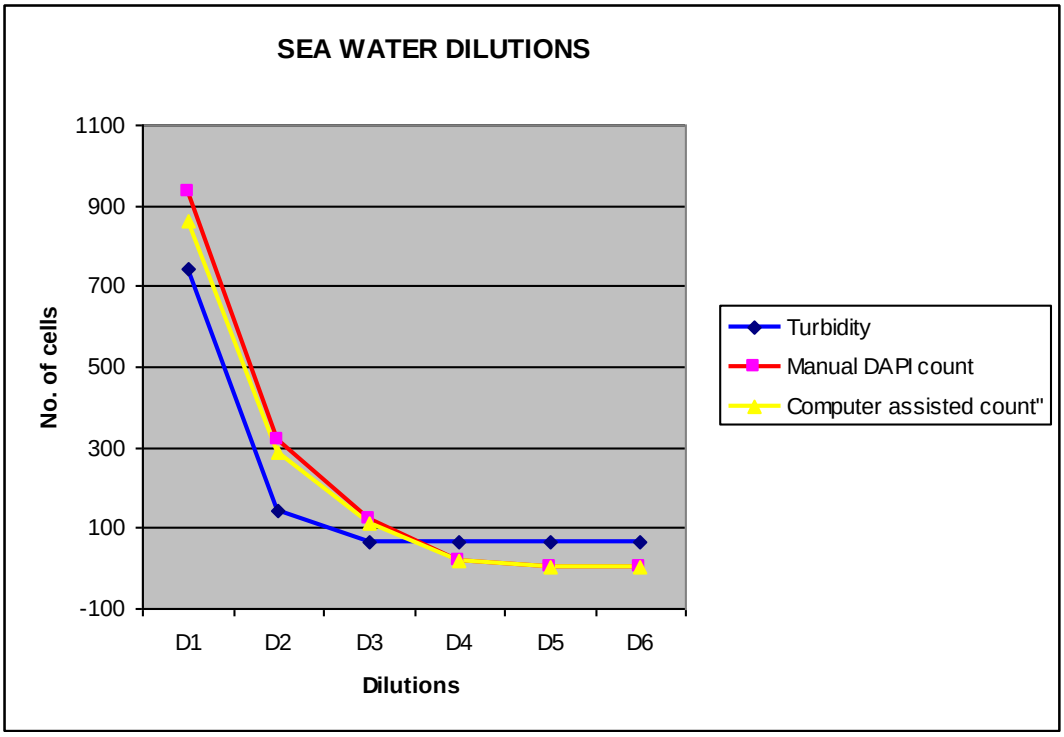


Figure 15 – Comparison between DAPI count and turbidity in sea water

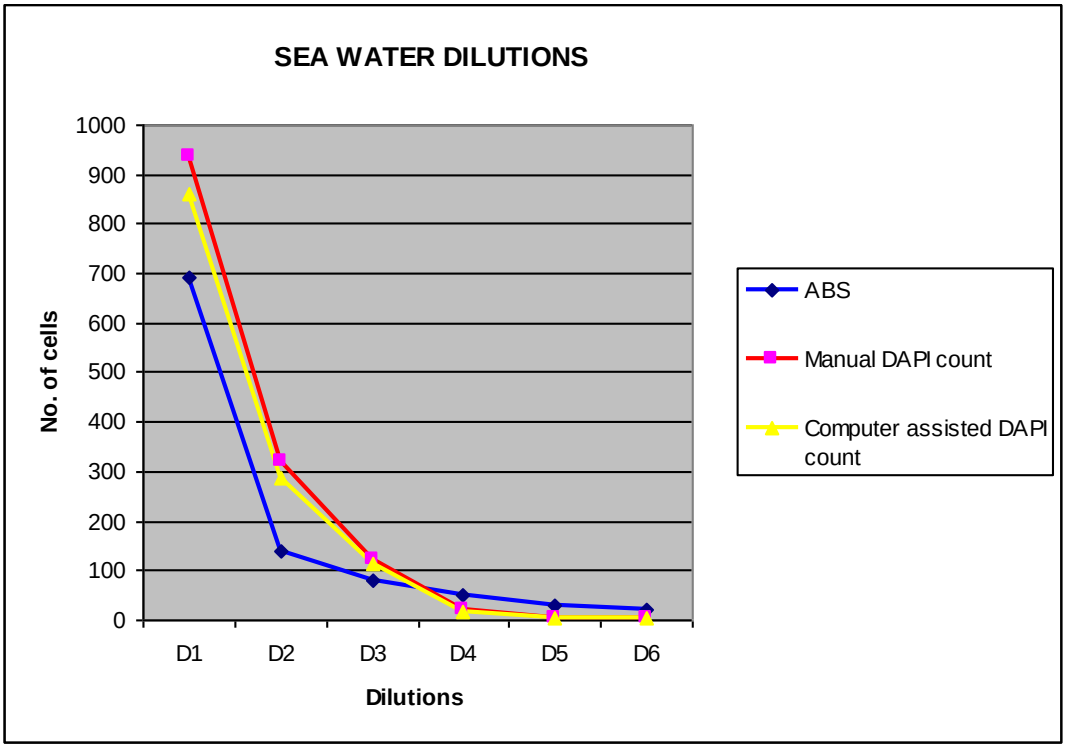


Figure 16 – Comparison between DAPI count and ABS in sea water

4.3 Plate count

Plate counts are based on the ability of bacteria in a sample to grow on a defined nutrient medium. When bacteria grow on a nutrient, they form distinct colonies. Theoretically, a colony is derived from a single bacteria cell and each individual bacterial cell will develop a colony which can be counted. According some authors, plate counting underestimates the number of bacteria because some groups of bacteria form only one colony. Another reason that plate counts can be too low is that the bacteria are in a starved state and cannot grow on rich nutrient media and also rich laboratory media are toxic to bacteria adapted to living in pure water systems.

Table 4 - Pseudomonas putida dilutions plate count

	D1	D2	D3	D4	D5	D6
A1	TMTC	TMTC	TMTC	1856	702	49
A2	TMTC	TMTC	TMTC	2164	998	75
A3	TMTC	TMTC	TMTC	1839	1048	103
Average	-	-	-	**1953**	**916**	**75.66667**
St.dev	-	-	-	**182.9289**	**187.008**	**27.00617**
St.error	-	-	-	**105.6141**	**107.9691**	**15.59202**

Table 5 - Sea water dilutions plate count

	D1	D2	D3	D4	D5	D6
A1	1687	812	196	96	26	17
A2	1524	710	287	118	23	21
A3	1326	581	372	136	19	25
Average	**1512.333**	**701**	**285**	**116.6667**	**22.66667**	**21**
St.dev	**180.7826**	**115.7627**	**88.01704**	**20.03331**	**3.511885**	**4**
St.error	**104.3749**	**66.83562**	**50.81666**	**11.56623**	**2.027588**	**2.309401**

In the tables above there are the data collected from direct plate count of sea water and pseudomonas cultures on Petri dishes. Al three triplicates cultures show that in case of pseudomonas pure culture there are TMTC colonies from dilution 10^{-3} to 10^{-1} and in the case of sea water cultures the manual count could be done until dilution 10^{-1}.

Although sea water cultures must have a high number of colonies than in pure culture, the results reveals that the number is low, probably because of growing media used which acted as a poison media for certain bacteria in the water sample and also because of growing environment such as air or temperature.

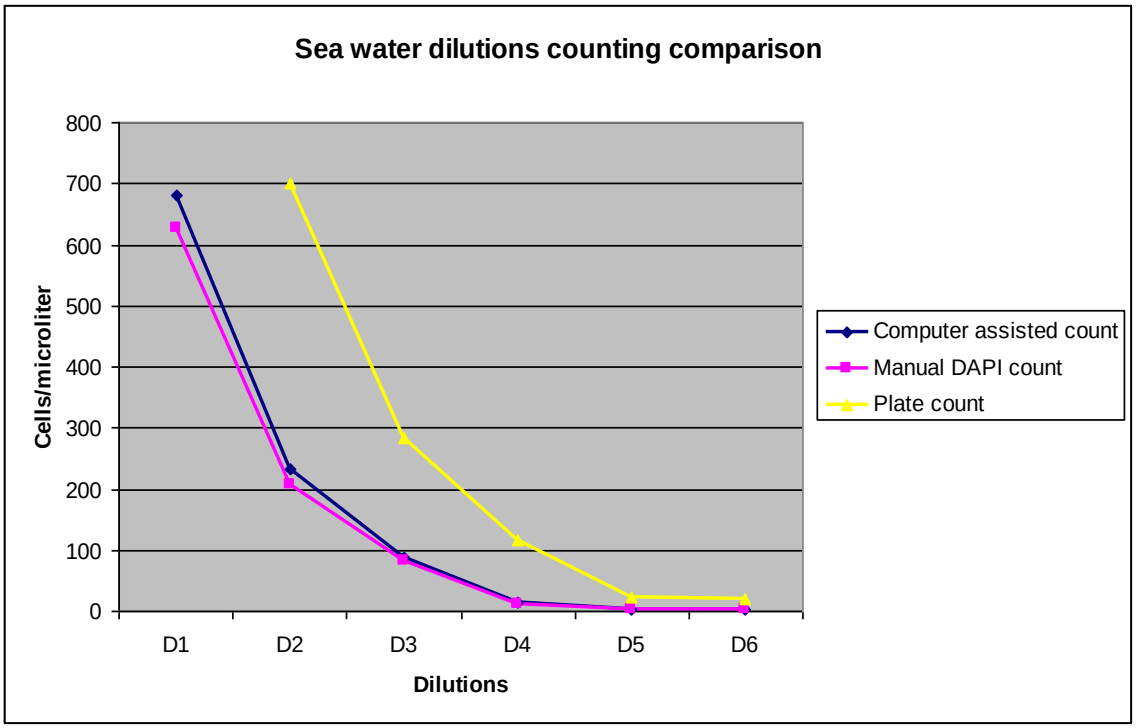


Figure 17 – Comparison between counting techniques in sea water dilutions

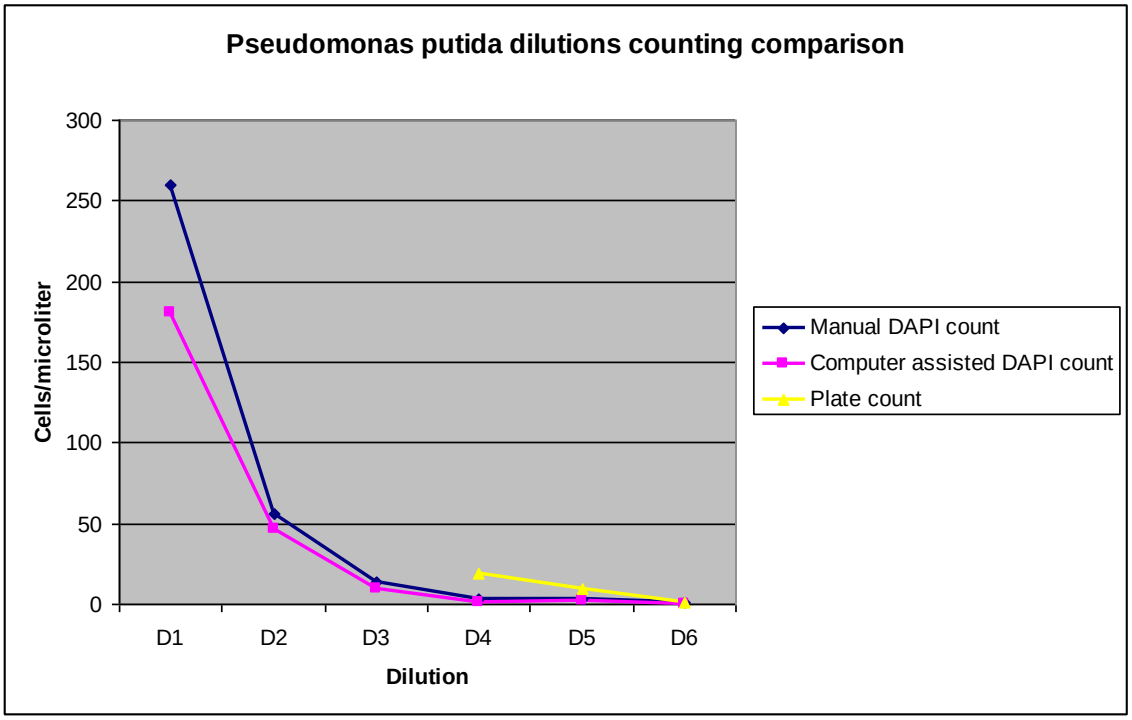


Figure 18 – Comparison between counting techniques in *Pseudomonas* dilutions

Figure 17 and 18 show the comparison between manual DAPI counts, computer assisted counts and plate direct counts at the same concentration in the diluted samples, given in number of cells / µl. The data are analyzed by comparing the values for each count at corresponding dilution and reveals that the plate count is not relevant because lack of data from pseudomonas plate count and in case of sea water, the results are far from the DAPI counting, both manual and computer assisted.

4.4. Manual count versus computer assisted count

The correlation between DAPI direct count and computer assisted count are analyzed by comparing the values of S_E and S_D of *Pseudomonas putida* pure culture and sea water dilutions for each range of diluted sample (fig. 19, fig. 20, fig 21and fig.22). The figures show also the red line for target correlation.

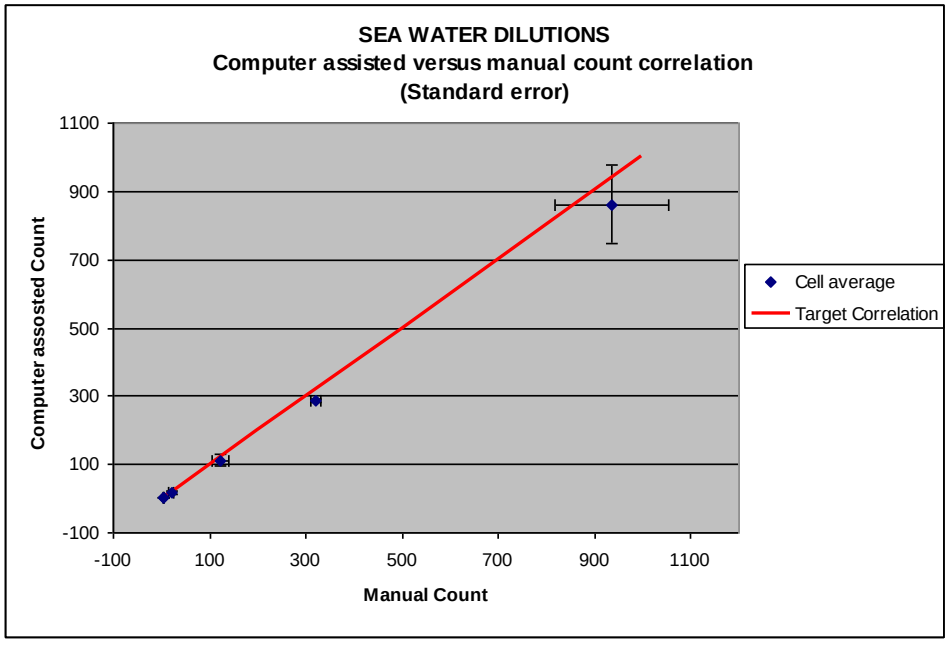


Figure 19 – Computer assisted versus manual count in SW dilutions (S_E)

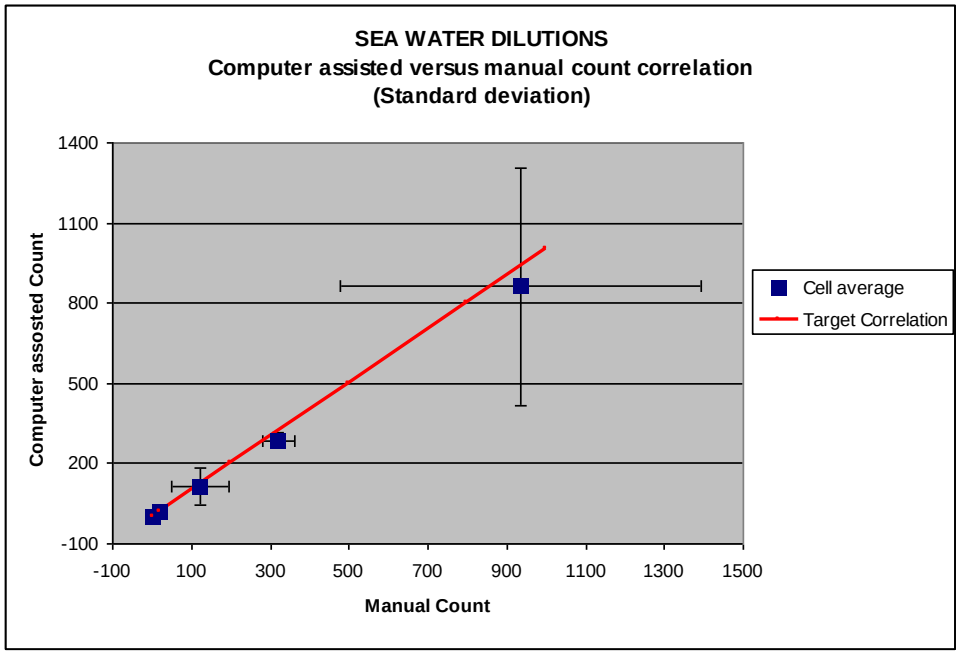


Figure 20 – Computer assisted versus manual count in sea water dilutions (S_D)

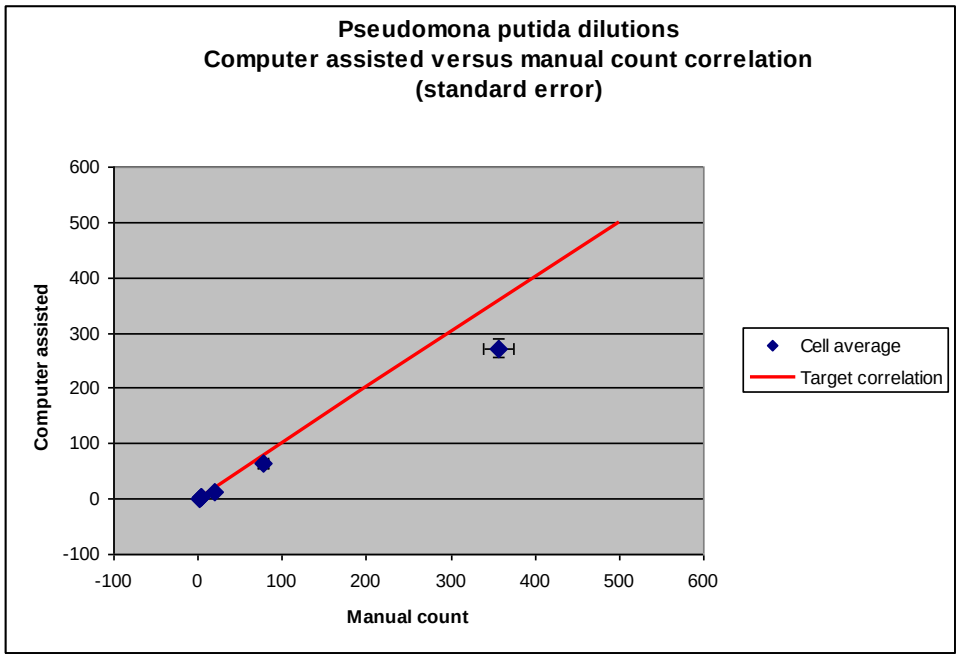


Figure 21 – Computer assisted versus manual count in *Pseudomonas putida* dilutions (S_E)

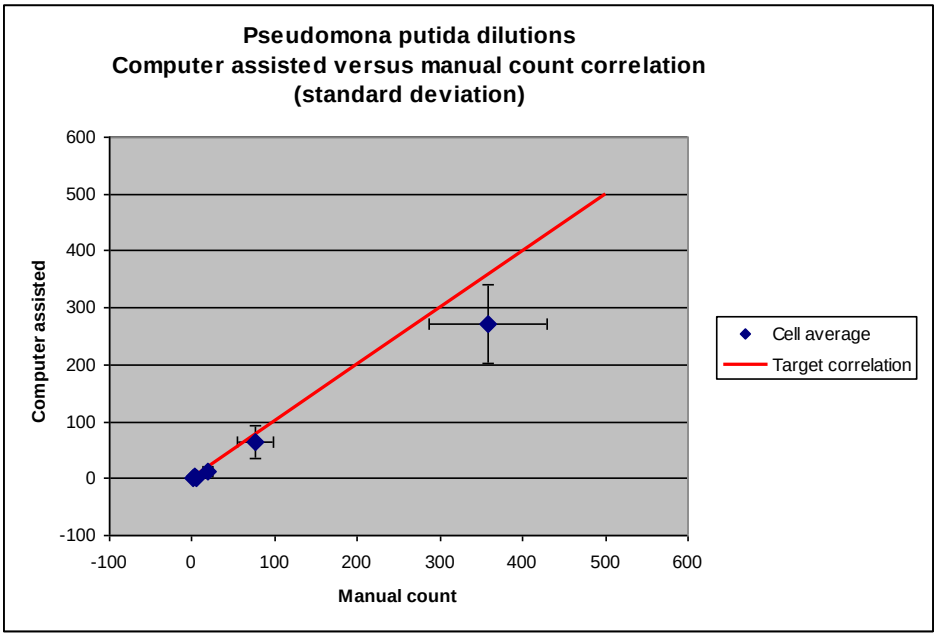


Figure 22 – Computer assisted versus manual count in *Pseudomonas putida* dilutions (S_D)

Figures 19 and 21 analysis shows that S_E in both *Pseudomonas putida* pure culture and sea water culture are low for DAPI computer assisted count than DAPI direct count. Because standard error is used to refer to an estimate of unknown quantity (of cells in this case), it can be deduced that computer assisted count technique is accurate than direct DAPI count in both pure culture and sea water samples.

Figure 20 and figure 22 show that standard deviation is lower in computer assisted DAPI count than in direct DAPI count. A low standard deviation indicates that the data points tend to be very close to the mean[3], whereas high standard deviation indicates that the data is spread out over a large range of values. This lead to the same finding that computer assisted count are accurate than direct count in

[3] The expected value of a random variable, average

both pure culture and sea water samples.

Also it can be seen that the accuracy in estimation of DAPI cells in pure culture of *Pseudomonas putida* it's higher than in sea water culture because there is only one species in pure culture and distribution in the sample is uniform.

4.5. Errors

Obvious there was some errors during the experiment, human but also due to the technique and procedures.
One possible source of errors is human factor which can modify accidentally the sample content and registration of data related to the samples, errors in manipulation of equipment and saving essential data, errors in doing filtration, preparing the sample, for example type of oil used or amount of oil used on the slides.
Regarding the procedures and technique, the errors can occur in DAPI staining, filtration, manipulation of materials and instruments, the quality of filters, the quality of microscope, the quality of digital camera.

5. Conclusions

DAPI technique requires no special preparation of cell and can be used to stain dead cells as well as living cells and reveal bacteria in the samples. Application of DAPI on both pure culture of *Pseudomonas putida* and marine water show the bacterial distribution very clear when looking in the microscope ocular but not in the picture captured with digital camera VisiCam attached on the microscope.

The experiment shows that plating techniques which detect bacteria by counting colonies of on the plates fail to reveal the correct number of the cells both in sea water and pure culture of *Pseudomonas putida* and estimates lower numbers of bacteria in sea water. Many species in the marine water have different growth requirements and they not grow on the plate, most probably because in the experiment had been used only a single type of growing media and also because there was a single grow temperature, 30^0C.

Another reason that viable bacteria plate counts can be too low is that the bacteria are in a starved state and cannot grow on rich nutrient media. It seems that rich laboratory media like Agar used in the experiment can be toxic to bacteria adapted for living in sea water.

Experience is required with the microscope, sample preparation, to change camera software settings, amount of immersion oil, or type of oil in order to obtain pictures that are easily readable for Matlab.

A clean lens microscope equipped with a deep field objective must be used to obtain pictures which show all bacteria in the sample. If the depth field of microscope objective is limited, not all the bacteria on the

slide will appear in the captured image because are spread at different levels in immersion oil layer on the slide.
A very good quality digital camera with auto focus feature must be fitted on the microscope in order to obtain pictures that are very similar to the image seen in the ocular by the human eye and to improve the speed and quality of captured pictures.
An anti-fade reagent must be used for easy and rapid picture taking when prepare the slide with samples.

There are some differences between the manual DAPI count and computer assisted count using Matlab software and is clear that lots of cells in the picture captured with the camera did not become recognized by the Matlab, probably because of the light in the background and also possible because the Matlab software. The version of Matlab used for cell count was 7.8.0.347 (R2009b), 32 bit (win 32) but with some modifications made by IVAR scientists.
There are differences between the low abundance of bacteria in marine water revealed by plate counting method of viable bacteria and DAPI counting, direct and automatic, that is still a question that is not solved yet.

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Appendix 1
Matlab computer assisted count data

Pseudomonas 10^{-1} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	227	22	152	17	5.443903
2	349	24	227	98	7.521382
3	218	16	158	44	4.986259
4	287	0	0	3	0.006867
5	302	0	0	10	0.036616
6	190	0	0	19	0.073211
7	237	11	159	67	5.024899
8	220	3	120	97	3.767687
9	215	8	119	88	3.907758
10	257	41	181	35	7.241616
11	455	136	289	130	15.40283
12	249	26	197	26	6.91541
13	234	22	180	32	6.159106
14	329	58	205	66	8.713658
15	632	239	292	101	20.18709

Pseudomonas 10^{-2} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	36	0	10	26	0.416861
2	20	0	12	8	0.289451
3	38	1	16	21	0.564871
4	41	1	19	21	0.673983
5	75	7	59	9	1.984779
6	107	31	50	26	3.679075
7	97	1	87	9	2.380068
8	95	10	63	22	2.17637
9	35	0	23	12	0.599879
10	85	2	50	33	1.524495
11	60	1	53	6	1.454598
12	40	4	21	15	0.778988
13	83	6	64	13	2.037033
14	105	20	68	17	3.00398

15	48	3	27	18	1.055995

Pseudomonas 10^{-3} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	17	0	8	9	0.23043
2	8	1	6	1	0.2162
3	11	1	6	4	0.213045
4	21	4	12	5	0.552785
5	16	1	10	5	0.375522
6	10	2	4	4	0.169086
7	10	0	8	2	0.255038
8	7	0	5	2	0.174762
9	9	0	8	1	0.241662
10	15	0	9	6	0.37195
11	14	0	2	12	0.127291
12	12	1	8	3	0.295048
13	18	0	13	5	0.421644
14	7	1	3	3	0.144497
15	11	1	8	2	0.319696

Pseudomonas 10^{-4} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	1	0	1	0	0.027566
2	2	1	1	0	0.082955
3	3	2	1	0	0.097602
4	3	0	2	1	0.056918
5	3	0	1	2	0.025264
6	2	0	0	2	0.012543
7	3	0	3	0	0.093613
8	1	0	1	0	0.019826
9	3	0	1	2	0.0386
10	1	0	1	0	0.026693
11	1	0	1	0	0.024033
12	1	0	1	0	0.034571
13	1	0	1	0	0.018099
14	5	2	1	2	0.100321
15	1	0	1	0	0.02439

Pseudomonas 10^{-5} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	3	1	1	1	0.163966
2	1	0	1	0	0.027705
3	6	0	4	2	0.152376
4	4	0	2	2	0.03995
5	0	0	0	0	0
6	1	0	1	0	0.028737
7	6	2	2	2	0.16734
8	2	2	0	0	0.052472
9	0	0	0	0	0
10	3	0	2	1	0.056501
11	4	0	4	0	0.105302
12	2	0	1	1	0.024271
13	0	0	0	0	0
14	5	4	1	0	0.332179
15	3	0	2	1	0.048364

Pseudomonas 10^{-6} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	0	0	0	0	0
2	2	0	1	1	0.027486
3	0	0	0	0	0
4	2	0	0	2	0.013694
5	0	0	0	0	0
6	0	0	0	0	0
7	0	0	0	0	0
8	0	0	0	0	0
9	1	0	1	0	0.046439
10	0	0	0	0	0
11	0	0	0	0	0
12	0	0	0	0	0
13	1	0	1	0	0.016353
14	1	0	1	0	0.033063
15	1	0	1	0	0.015817

Sea water 10^{-1} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	866	22	56	788	5.015988
2	505	17	24	464	2.788455
3	663	12	32	619	3.113489
4	752	19	49	684	4.351888
5	83	8	2	73	0.506465
6	1612	240	192	1180	21.26361
7	1260	148	156	956	14.54872
8	1285	181	141	963	16.84841
9	1120	180	131	809	15.9345
10	800	75	90	635	8.318072
11	368	2	15	351	1.294165
12	1097	155	111	831	12.96042
13	1034	161	116	757	14.14661
14	1316	73	120	1123	10.69883
15	157	2	8	147	0.724967

Sea water 10^{-2} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	310	24	73	213	4.016216
2	276	31	67	178	4.510078
3	291	15	92	184	4.375941
4	228	10	59	159	2.711671
5	285	21	79	185	3.879875
6	296	36	69	191	4.663209
7	331	23	70	238	4.081687
8	267	6	47	214	2.562748
9	292	20	62	210	3.566827
10	236	13	51	172	2.766009
11	297	28	73	196	4.213662
12	308	16	70	222	3.751949
13	293	17	66	210	3.46097
14	292	21	65	206	3.966105
15	299	10	71	218	3.455472

Sea water 10^{-3} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	78	0	4	74	0.267363
2	211	0	4	207	0.901615
3	134	0	4	130	0.565248
4	190	0	4	186	0.707523
5	253	0	7	246	1.060381
6	122	0	5	117	0.587376
7	140	0	2	138	0.529724
8	144	0	5	139	0.56886
9	47	0	1	46	0.187126
10	62	0	3	59	0.247497
11	30	0	2	28	0.144001
12	49	0	3	46	0.241881
13	143	0	4	139	0.506366
14	27	0	3	24	0.16599
15	64	0	3	61	0.292865

Sea water 10^{-4} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	50	0	2	48	0.260337
2	42	0	1	41	0.190817
3	35	0	1	34	0.155432
4	9	0	1	8	0.073945
5	15	0	6	9	0.192981
6	8	0	4	4	0.104607
7	5	0	3	2	0.092839
8	7	0	2	5	0.073668
9	12	0	4	8	0.138305
10	16	0	8	8	0.230827
11	9	1	5	3	0.22011
12	6	0	2	4	0.061264
13	12	0	2	10	0.105996
14	15	1	8	6	0.327019
15	12	0	6	6	0.177382

Sea water 10^{-5} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	1	0	0	1	0.005775
2	2	0	0	2	0.010935
3	4	0	1	3	0.035087
4	4	0	1	3	0.0361
5	4	0	1	3	0.036814
6	2	0	0	2	0.01161
7	4	0	0	4	0.022247
8	10	0	0	10	0.068964
9	8	0	1	7	0.049237
10	8	0	1	7	0.049575
11	8	0	1	7	0.049873
12	1	0	0	1	0.003116
13	2	0	0	2	0.013078
14	0	0	0	0	0
15	0	0	0	0	0

Sea water 10^{-6} MATLAB count data

Picture No.	Number estimated	Number small	Number medium	Number big	Cover
1	1	0	0	1	0.005775
2	2	0	0	2	0.010935
3	4	0	1	3	0.035087
4	4	0	1	3	0.0361
5	4	0	1	3	0.036814
6	2	0	0	2	0.01161
7	4	0	0	4	0.022247
8	10	0	0	10	0.068964
9	8	0	1	7	0.049237
10	8	0	1	7	0.049575
11	8	0	1	7	0.049873
12	1	0	0	1	0.003116
13	2	0	0	2	0.013078
14	0	0	0	0	0
15	0	0	0	0	0

Appendix 2
Plate direct count data

Pseudomonas putida dilutions plate count

	D1	D2	D3	D4	D5	D6
A1	TMTC	TMTC	TMTC	1856	702	49
A2	TMTC	TMTC	TMTC	2164	998	75
A3	TMTC	TMTC	TMTC	1839	1048	103
Average	-	-	-	**1953**	**916**	**75.66667**
St.dev	-	-	-	**182.9289**	**187.008**	**27.00617**
S_E	-	-	-	**105.6141**	**107.9691**	**15.59202**

Sea water dilutions plate count

	D1	D2	D3	D4	D5	D6
A1	1687	812	196	96	26	17
A2	1524	710	287	118	23	21
A3	1326	581	372	136	19	25
Average	**1512.333**	**701**	**285**	**116.6667**	**22.66667**	**21**
St.dev	**180.7826**	**115.7627**	**88.01704**	**20.03331**	**3.511885**	**4**
S_E	**104.3749**	**66.83562**	**50.81666**	**11.56623**	**2.027588**	**2.309401**

Appendix 3
DAPI direct count

Pseudomonas putida dilutions manual count

Pseudomonas putida manual count						
No. of picture	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
1	253	61	22	2	3	1
2	412	69	15	5	2	4
3	324	48	16	4	9	1
4	356	43	31	5	7	2
5	278	50	25	7	0	2
6	370	96	13	5	1	0
7	426	68	18	6	6	0
8	465	112	15	2	2	0
9	315	89	17	8	0	3
10	264	93	18	2	4	0
11	480	70	21	2	8	0
12	401	60	18	2	3	0
13	372	96	34	1	0	1
14	282	97	11	11	11	2
15	363	101	19	1	3	2

Sea water dilutions manual count

Sea water dilutions manual count						
No. of picture	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
1	1032	387	82	59	2	1
2	625	402	212	49	3	2
3	787	314	145	39	5	5
4	787	267	210	12	4	5
5	104	299	267	19	4	7
6	1734	311	134	11	3	2
7	1348	324	156	7	5	3
8	1306	286	151	8	11	4
9	1178	322	53	15	9	10
10	844	245	76	18	9	3
11	380	324	38	13	12	1
12	1167	356	52	9	1	4
13	1098	327	149	14	3	2
14	1392	336	37	16	0	0
15	245	312	74	15	0	0

Absorbance at 600nm and turbidity

Appendix 4
Absorbance & Turbidity

	Turbidity		Absorbance	
	Pseudomonas putida	**Sea water**	**Pseudomonas putida**	**Sea water**
Dilution 0	59.6	145.14	0.784	0.785
Dilution 1	7.44	92.68	0.069	0.05
Dilution 2	1.41	25.67	0.014	0.04
Dilution 3	0.65	12.63	0.008	0.016
Dilution 4	0.64	11.58	0.002	0.015
Dilution 5	0.65	4.7	0.005	0.016
Dilution 6	0.64	4.1	0.003	0.014

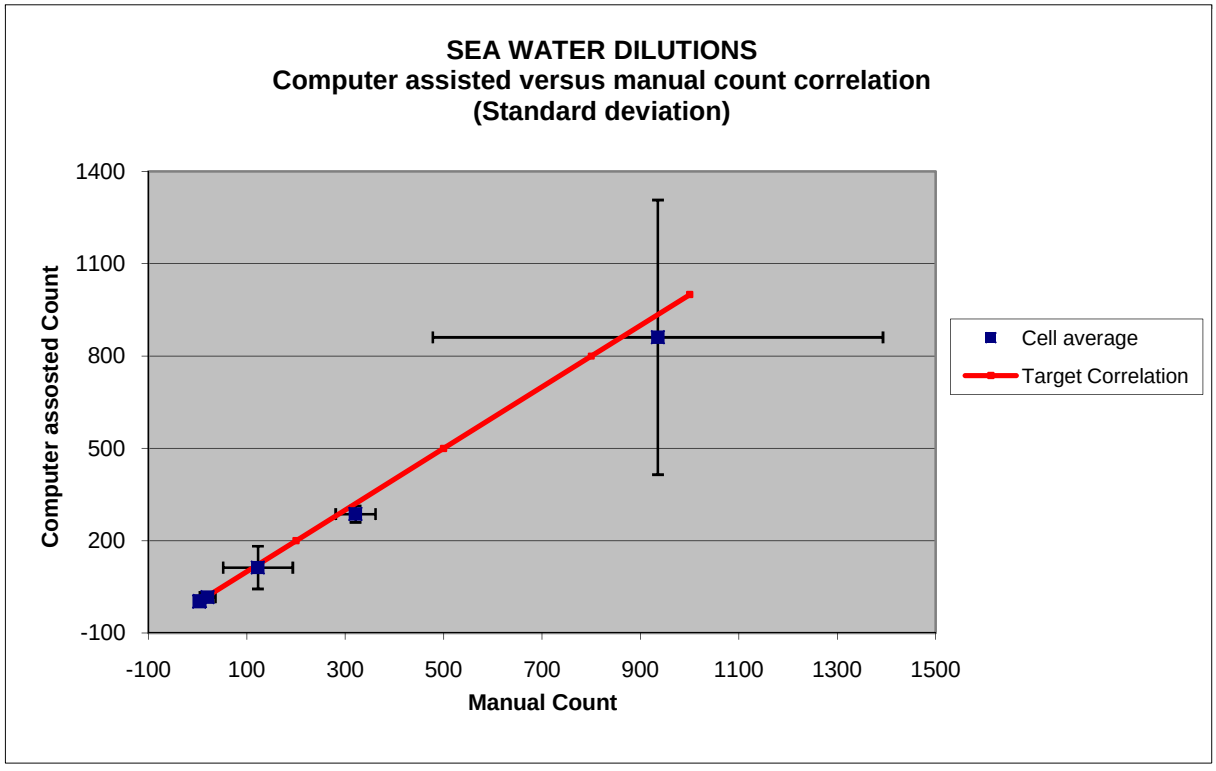
Sea Water Count

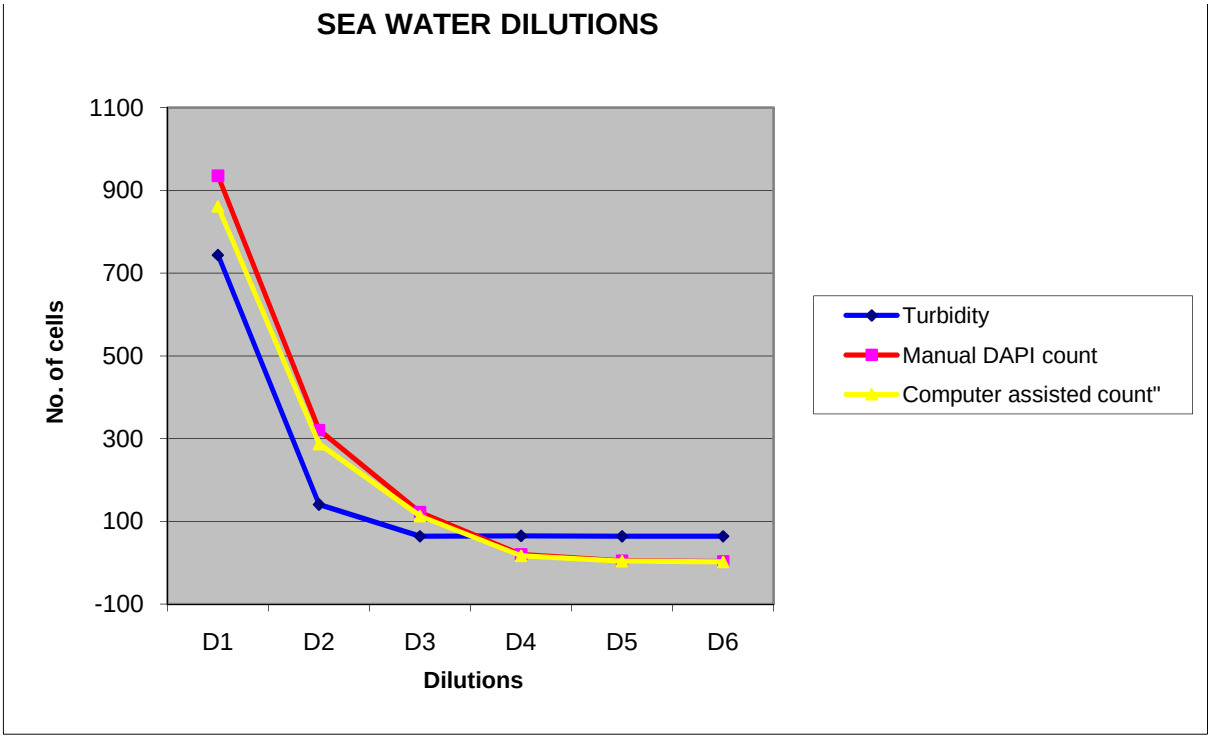
Manual count

Computer

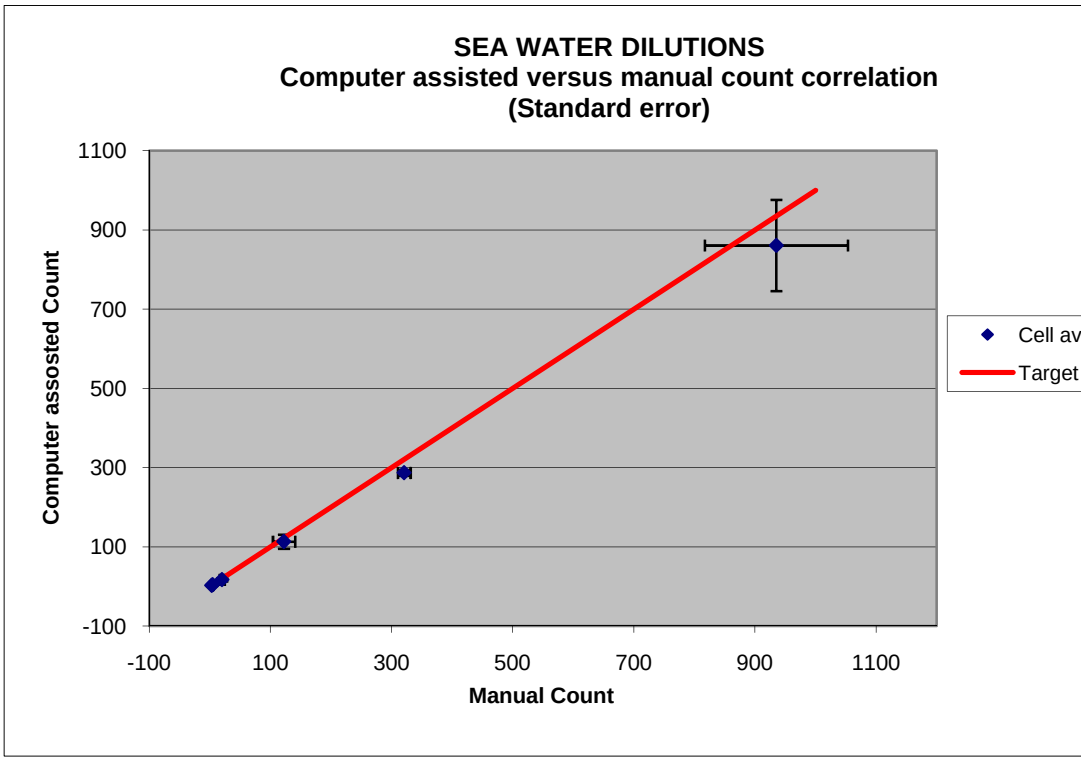
	D1	D2	D3	D4	D5	D6	
	1032	387	82	59	2	1	
	625	402	212	49	3	2	
	787	314	145	39	5	5	
	787	267	210	12	4	5	
	104	299	267	19	4	7	
	1734	311	134	11	3	2	
	1348	324	156	7	5	3	
	1306	286	151	8	11	4	
	1178	322	53	15	9	10	
	844	245	76	18	9	3	
	380	324	38	13	12	1	
	1167	356	52	9	1	4	
	1098	327	149	14	3	2	
	1392	336	37	16	0	0	
	245	312	74	15	0	0	
Average	**935,1333**	**320,8**	**122,4**	**20,26667**	**4,733333**	**3,266667**	**Average**
St.Dev	**457,2578**	**40,57832**	**70,82554**	**15,71381**	**3,825976**	**2,711527**	**St.Dev**
St.Error	**118,0635**	**10,47728**	**18,28708**	**4,057288**	**0,987863**	**0,700113**	**St.Error**
ABS	**0,069**	**0,014**	**0,008**	**0,005**	**0,003**	**0,002**	
Turb.	**7,44**	**1,41**	**0,64**	**0,65**	**0,64**	**0,64**	

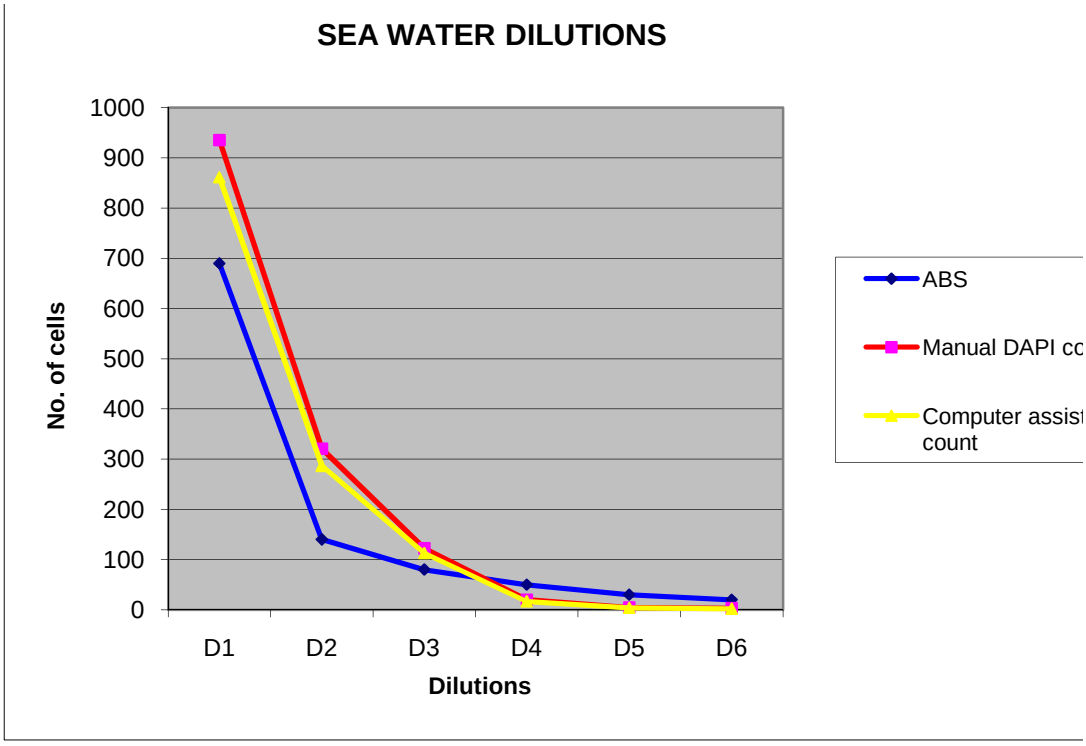
ABS	690	140	80	50	30	20
TURB	744	141	64	65	64	64



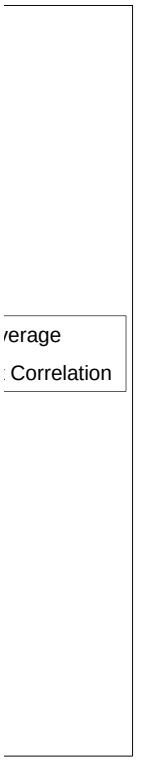

SEA WATER DILUTIONS
1100
900
700
500
300
100
-100
No. of cells
D1
D2
D3
D4
D5
D6
Dilutions
Turbidity
Manual DAPI count
Computer assisted count"

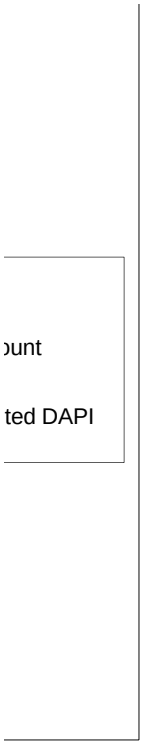
assisted count						Correlation line		
D1	D2	D3	D4	D5	D6	1	20	200
866	310	78	50	1	0	1	20	200
505	276	211	42	2	1			
663	291	134	35	4	3			
752	228	190	9	4	3			
83	285	253	15	4	4			
1612	296	122	8	2	1			
1260	331	140	5	4	2			
1285	267	144	7	10	3			
1120	292	47	12	8	8			
800	236	62	16	8	1			
368	297	30	9	8	5			
1097	308	49	6	1	0			
1034	293	143	12	2	2			
1316	292	27	15	0	0			
157	299	64	12	0	0			
861,2	**286,7333**	**112,9333**	**16,8667**	**3,86667**	**2,2**			
446,2722	**26,6444**	**69,28664**	**13,87632**	**3,226379**	**2,242448**			
115,227	**6,879553**	**17,88973**	**3,582852**	**0,833048**	**0,578997**			




SEA WATER DILUTIONS
1000
900
800
700
600
500
400
300
200
100
0
No. of cells
D1
D2
D3
D4
D5
D6
Dilutions
ABS
Manual DAPI cc
Computer assist
count

500	800	1000	1500
500	800	1000	1500

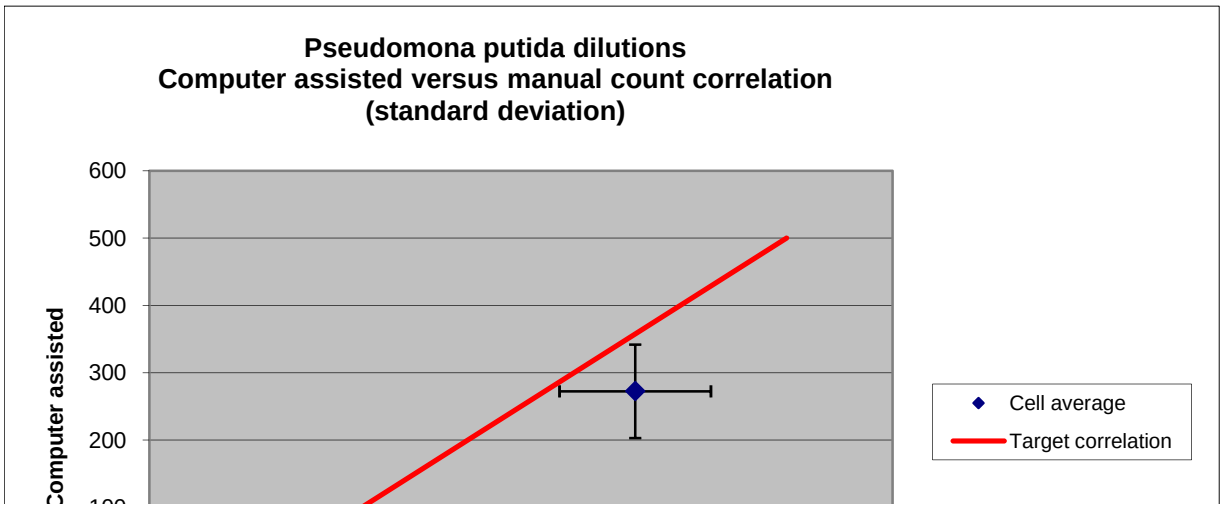


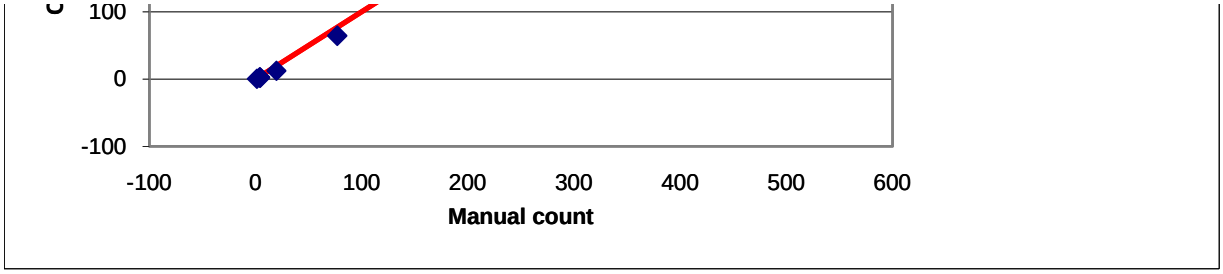

ount
ted DAPI

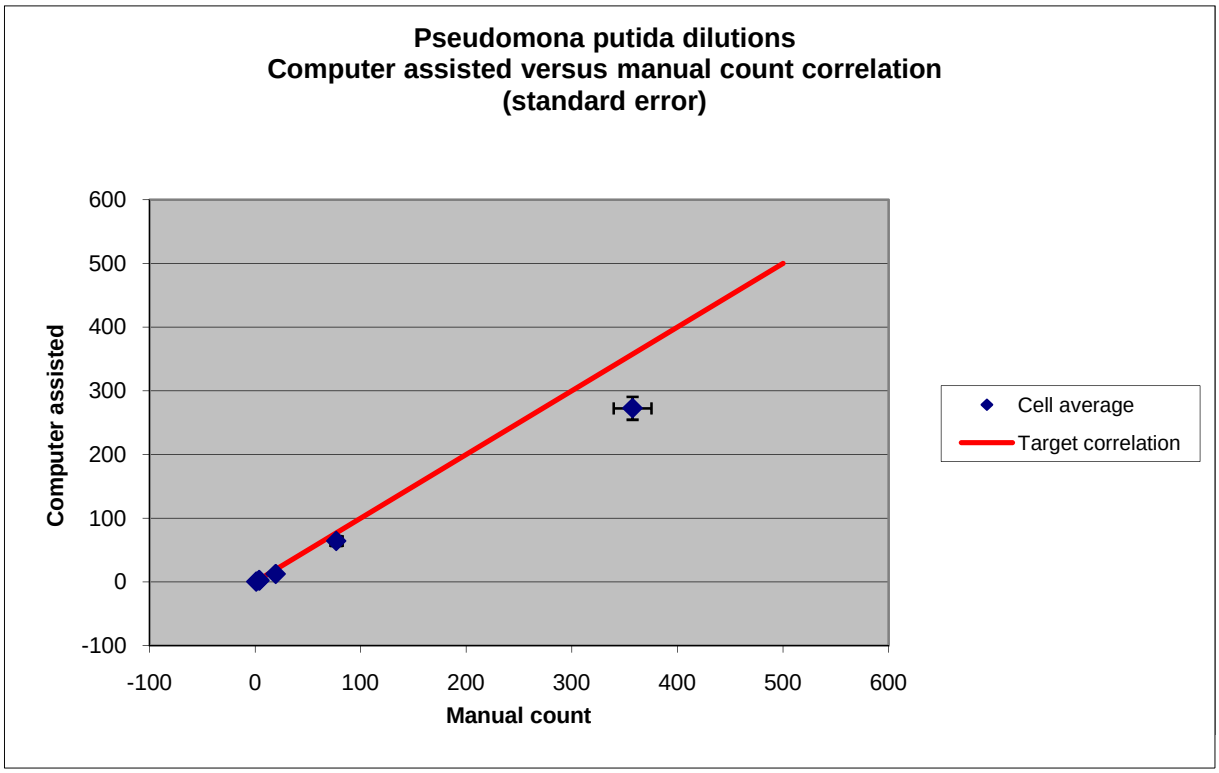
Pseudomonas putida count

Manual Count	D1	D2	D3	D4	D5	D6	Computer
1	253	61	22	2	3	1	**1**
2	412	69	15	5	2	4	**2**
3	324	48	16	4	9	1	**3**
4	356	43	31	5	7	2	**4**
5	278	50	25	7	0	2	**5**
6	370	96	13	5	1	0	**6**
7	426	68	18	6	6	0	**7**
8	465	112	15	2	2	0	**8**
9	315	89	17	8	0	3	**9**
10	264	93	18	2	4	0	**10**
11	480	70	21	2	8	0	**11**
12	401	60	18	2	3	0	**12**
13	372	96	34	1	0	1	**13**
14	282	97	11	11	11	2	**14**
15	363	101	19	1	3	2	**15**
Average	**357,4**	**76,86667**	**19,53333**	**4,2**	**3,933333**	**1,2**	**Average**
St.dev	**71,27793**	**22,01904**	**6,334336**	**2,908117**	**3,494213**	**1,264911**	**St.dev**
St.error	**17,89218**	**5,685292**	**1,635518**	**0,750873**	**0,902202**	**0,326599**	**St.error**
ABS	**0,069**	**0,014**	**0,008**	**0,002**	**0,005**	**0,003**	**ABS**
Turb.	**7,44**	**1,41**	**0,64**	**0,65**	**0,64**	**0,64**	**Turb.**

ABS	690	140	80	20	50	30	**ABS**
TURB	744	141	64	65	64	64	TURB







-50 0 20 50 200 400 500

count

D1	D2	D3	D4	D5	D6
227	36	17	1	3	0
349	20	8	2	1	2
218	38	11	3	6	0
287	41	21	3	4	2
302	75	16	3	0	0
190	107	10	2	1	0
237	97	10	3	6	0
220	95	7	1	2	0
215	35	9	3	0	1
257	85	15	1	3	0
455	60	14	1	4	0
249	40	12	1	2	0
234	83	18	1	0	1
329	105	7	5	5	1
321	48	11	1	3	1

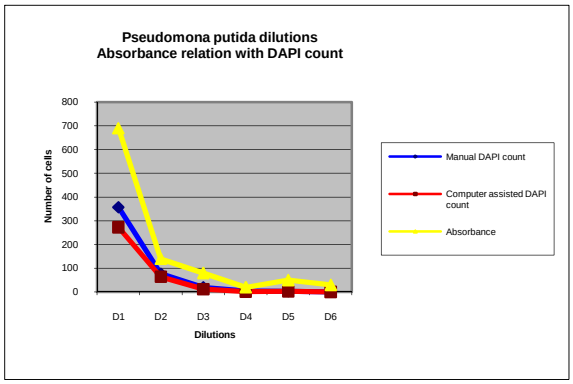
Correlation line

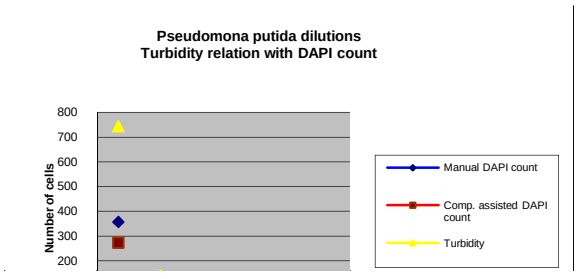
1	20
1	20

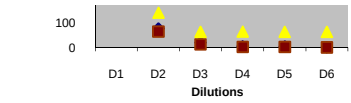
	PP Turb
D0	59,6
D1	7,44
D2	1,41
D3	0,65
D4	0,64
D5	0,65
D6	0,64

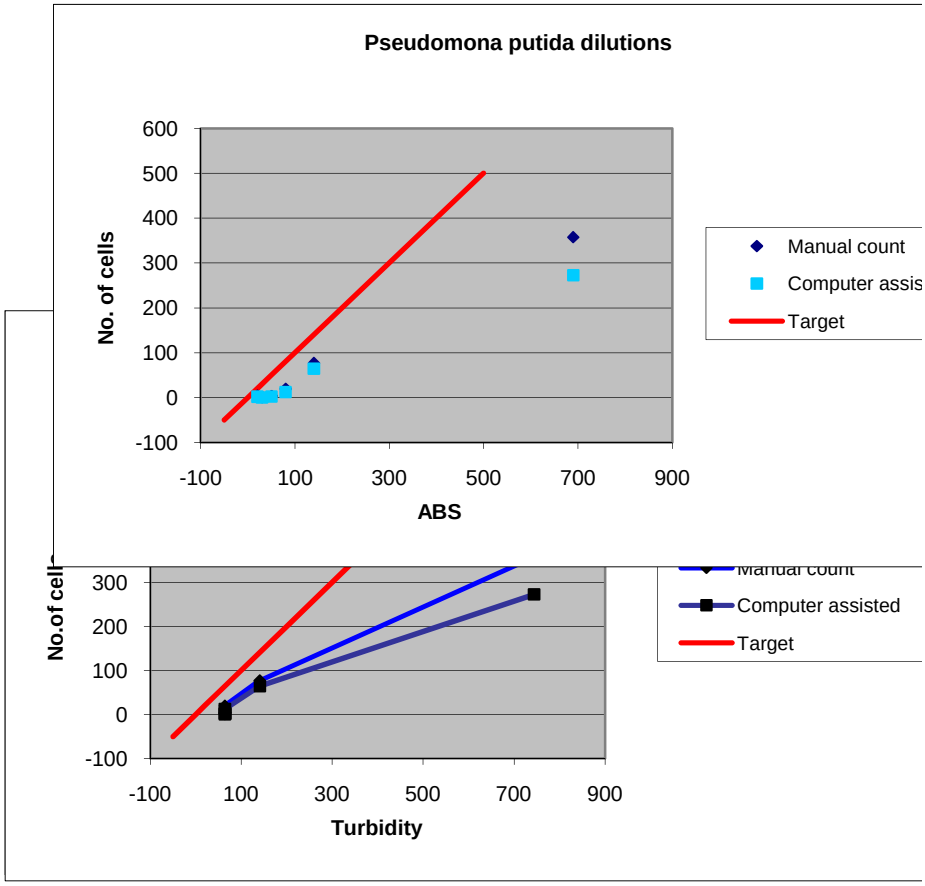
272,6667	**64,33333**	**12,4**	**2,066667**	**2,666667**	**0,533333**
69,29612	**29,41736**	**4,256088**	**1,222799**	**2,058663**	**0,743223**
17,89218	**7,595529**	**1,098917**	**0,315725**	**0,531545**	**0,191899**
0,069	**0,014**	**0,008**	**0,002**	**0,005**	**0,003**
7,44	**1,41**	**0,64**	**0,65**	**0,64**	**0,64**

690	140	80	20	50	30
744	141	64	65	64	64






100
0
D1
D2
D3
D4
D5
D6
Dilutions


Pseudomona putida dilutions
600
500
400
300
200
100
0
-100
No. of cells
-100
100
300
500
700
900
ABS
Manual count
Computer assis
Target

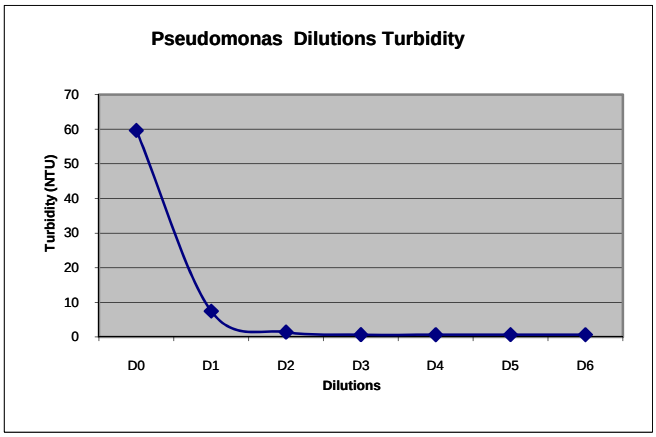
300
200
100
0
-100
-100
100
300
500
700
900
Turbidity
Computer assisted
Target

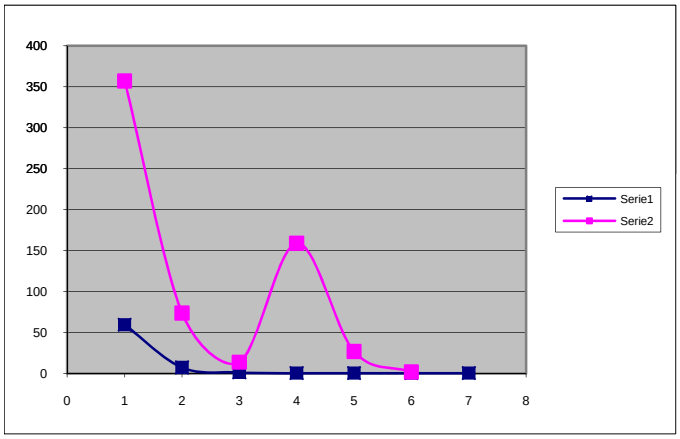
200	500
200	500

SW Turb	ABS PP	ABS SW
145,14	0,784	0,785
92,68	0,069	0,05
25,67	0,014	0,04
12,63	0,008	0,016
11,58	0,002	0,015
4,7	0,005	0,016
4,1	0,003	0,014

sted

	PP Turb	SW Turb	ABS PP	ABS SW
D0	59,6	145,14	0,784	0,785
D1	7,44	92,68	0,069	0,05
D2	1,41	25,67	0,014	0,04
D3	0,65	12,63	0,008	0,016
D4	0,64	11,58	0,002	0,015
D5	0,65	4,7	0,005	0,016
D6	0,64	4,1	0,003	0,014





Pseudomonas

D1 = 10 MINUS 1,……

	Nest
D1	357
D2	74
D3	14
D4	159
D5	27
D6	2

Sea Water com

	Nest
D1	1721
D2	344
D3	375
D4	702
D5	725
D6	4

pure culture computer assisted count

D6 = 10 minus 6

Nsmall	Nmed	Nbig	Cover	stdNest	stdNsmall	stdNmed	stdNbig	stdCover
105	198	54	8,282	183	120	46	34	4,162
15	45	14	1,687	40	23	21	8	1,052
3	8	4	0,325	5	3	3	2	0,161
72	3	84	2,058	319	144	4	175	4,076
11	2	15	0,386	77	30	1	47	0,93
0	1	1	0,029	2	1	1	2	0,028

puter assisted count

Nsmall	Nmed	Nbig	Cover	stdNest	stdNsmall	stdNmed	stdNbig	stdCover
957	117	647	29,846	260	249	35	143	5,816
86	87	171	5,628	15	17	8	16	0,373
28	50	298	3,236	155	37	18	109	1,419
136	27	540	5,336	261	44	10	224	1,428
140	34	551	5 589	243	42	7	203	1,476
0	0	4	0,031	3	0	1	3	0,024

PSEUDOMONA CELLS/microL

Average

D1	D2	D3	D4	D5	D6
247,8667	**64,33333**	**12,4**	**2,066667**	**2,666667**	**0,533333**
357,4	**76,86667**	**19,53333**	**4,2**	**3,933333**	**1,2**
0,72685	**0,72685**	**0,72685**	**0,72685**	**0,72685**	**0,72685**

Cells/microliter manual DAPI

259,7762	55,87054	14,1978	3,05277	2,858943	0,87222

Cells/microliter petri dishes

19,53	9,16	0,756

Cells/microliter computer assisted

D1	D2	D3	D4	D5	D6
180,1619	46,76068	9,01294	1,502157	1,938267	0,387653

no of cells x 0.72685=cells/microliter

Pseudomona

	D1
A1	TMTC
A2	TMTC
A3	TMTC

Average
St.dev
St.error

Sea water

	D1
A1	TMTC
A2	TMTC
A3	TMTC

Average
St.dev
St.error

Pseudomona manual DAPI count

	D1	D2	D3	D4	D5	D6
Average	**357,4**	**76,86667**	**19,53333**	**4,2**	**3,933333**	**1,2**

Pseudomona comp. assisted DAPI count

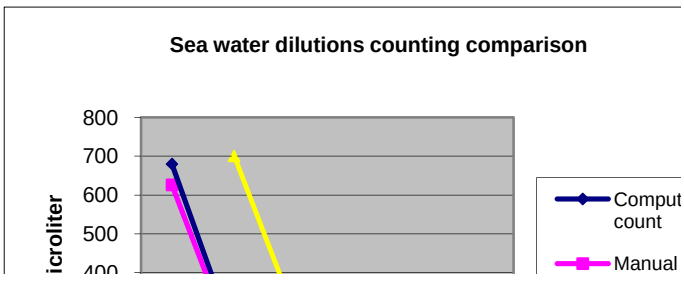
Average	**247,8667**	**64,33333**	**12,4**	**2,066667**	**2,666667**	**0,533333**

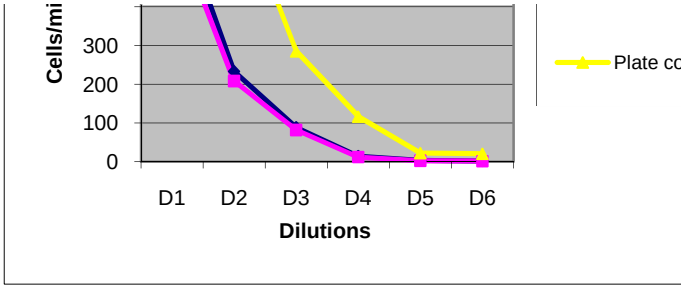
Sea water manual DAPI count

Average	861,2	286,7333	112,9333	16,8667	3,86667	2,2
Cell/micrl	625,9632	208,4121	82,08557	12,25956	2,810489	1,59907

Sea water DAPI comp. assisted countng

Average	935,1333	320,8	122,4	20,26667	4,733333	3,266667
Cell/micrl	679,7016	233,1735	88,96644	14,73083	3,440423	2,374377

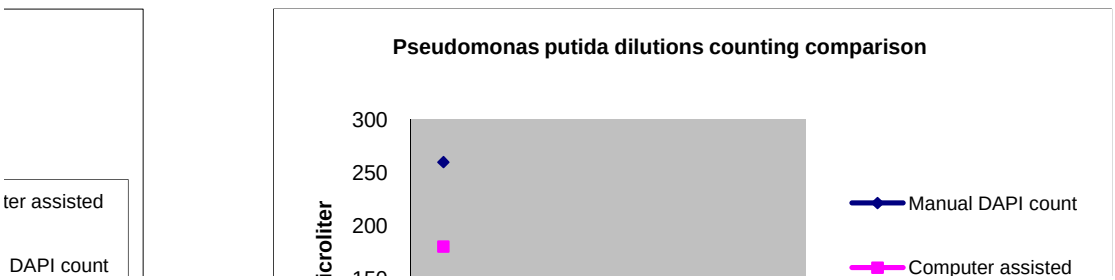


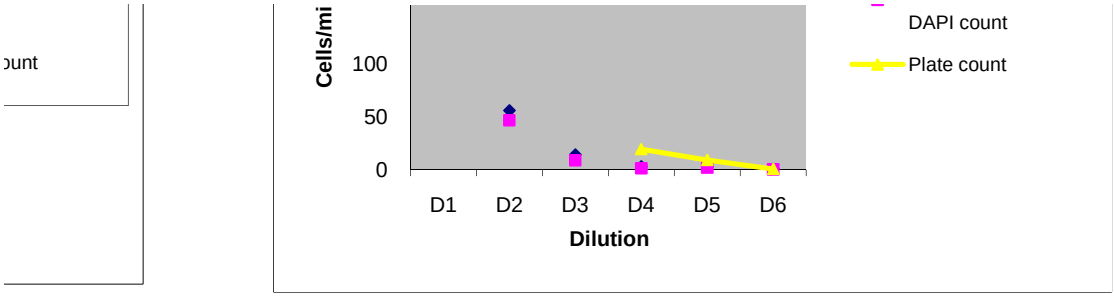

Cells/mi
300
200
100
0
D1
D2
D3
D4
D5
D6
Dilutions
Plate cc

number of colony for triplicate plates direct count

D2	D3	D4	D5	D6
TMTC	TMTC	1856	702	49
TMTC	TMTC	2164	998	75
TMTC	TMTC	1839	1048	103
		1953	**916**	**75,66667**
		182,9289	**187,008**	**27,00617**
		105,6141	**107,9691**	**15,59202**

D2	D3	D4	D5	D6
812	196	96	26	17
710	287	118	23	21
581	372	136	19	25
701	**285**	**116,6667**	**22,66667**	**21**
115,7627	**88,01704**	**20,03331**	**3,511885**	**4**
66,83562	**50,81666**	**11,56623**	**2,027588**	**2,309401**




ount
Cells/mi
100
50
0
D1
D2
D3
D4
D5
D6
Dilution
DAPI count
Plate count

PSEUDOMONA CELLS/microL

Average

D1	D2	D3	D4	D5	D6
247,8667	**64,33333**	**12,4**	**2,066667**	**2,666667**	**0,533333**
357,4	**76,86667**	**19,53333**	**4,2**	**3,933333**	**1,2**
0,72685	**0,72685**	**0,72685**	**0,72685**	**0,72685**	**0,72685**

Cells/microliter manual DAPI

259,7762	55,87054	14,1978	3,05277	2,858943	0,87222

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D1	D2	D3	D4	D5	D6
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no of cells x 0.72685=cells/microliter

Pseudomona

	D1
A1	TMTC
A2	TMTC
A3	TMTC

Average
St.dev
St.error

Sea water

	D1
A1	TMTC
A2	TMTC
A3	TMTC

Average
St.dev
St.error

Pseudomona manual DAPI count

	D1	D2	D3	D4	D5	D6
Average	**357,4**	**76,86667**	**19,53333**	**4,2**	**3,933333**	**1,2**

Pseudomona comp. assisted DAPI count

Average	**247,8667**	**64,33333**	**12,4**	**2,066667**	**2,666667**	**0,533333**

Sea water manual DAPI count

Average	861,2	286,7333	112,9333	16,8667	3,86667	2,2
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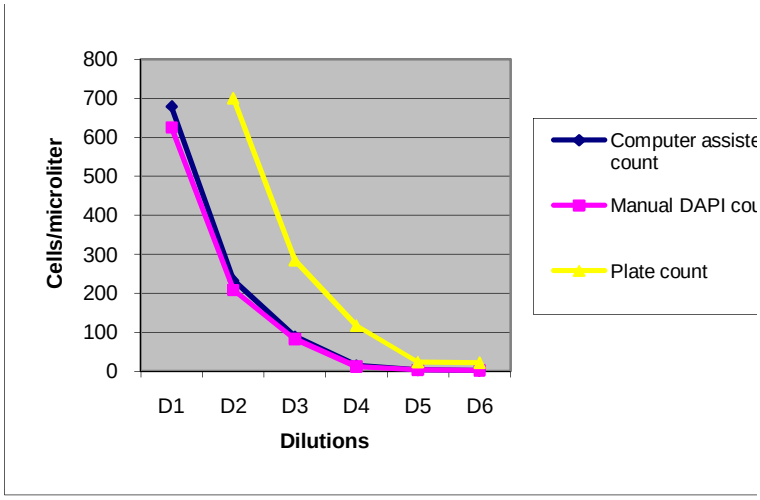
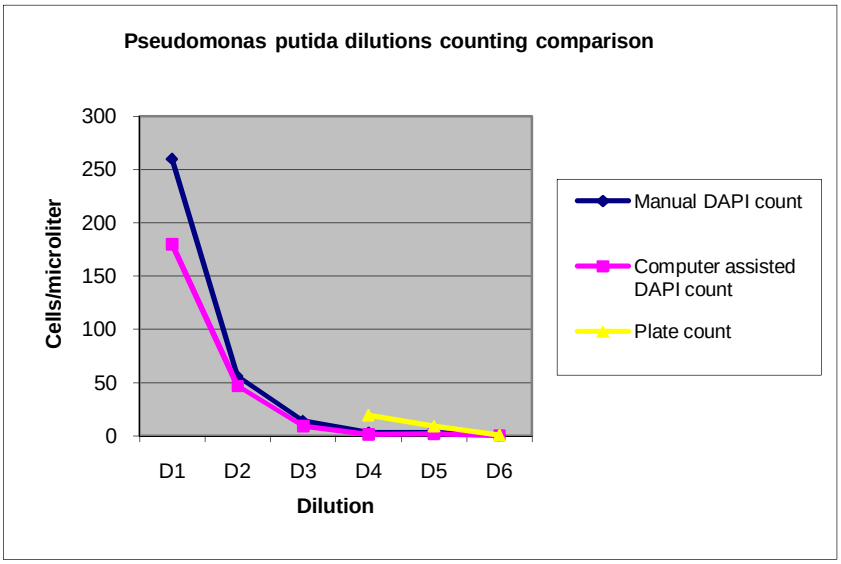
Cell/micrl	679,7016	233,1735	88,96644	14,73083	3,440423	2,374377


800
700
600
500
400
300
200
100
0
Cells/microliter
D1
D2
D3
D4
D5
D6
Dilutions
Computer assiste
count
Manual DAPI cou
Plate count

number of colony for triplicate plates direct count

D2	D3	D4	D5	D6
TMTC	TMTC	1856	702	49
TMTC	TMTC	2164	998	75
TMTC	TMTC	1839	1048	103
		1953	**916**	**75,66667**
		182,9289	**187,008**	**27,00617**
		105,6141	**107,9691**	**15,59202**

D2	D3	D4	D5	D6
812	196	96	26	17
710	287	118	23	21
581	372	136	19	25
701	**285**	**116,6667**	**22,66667**	**21**
115,7627	**88,01704**	**20,03331**	**3,511885**	**4**
66,83562	**50,81666**	**11,56623**	**2,027588**	**2,309401**


Pseudomonas putida dilutions counting comparison
300
250
200
150
100
50
0
Cells/microliter
D1
D2
D3
D4
D5
D6
Dilution
Manual DAPI count
Computer assisted DAPI count
Plate count