Photo-Fenton and Slow Sand Filtration coupling for hydroponics water reuse

Mª del Mar Micó Reche

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PHOTO-FENTON AND SLOW SAND FILTRATION COUPLING FOR HYDROPONICS WATER REUSE

Mª del Mar Micó Reche

Dirigida per: Dra. Carme Sans Mazón. Professora titular del departament d'Enginyeria Química de la Universitat de Barcelona.

Universitat de Barcelona

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OBJECTIVES

As part of the partnership between the Chemical Engineering Department of the University of Barcelona, and R+D Department of Acciona Agua, in the frame of CENIT-MEDIODIA Project. The main aim of this work is to study the suitability of a coupled system integrating photo-Fenton reaction and slow sand filtration, for the readaptation of hydroponic greenhouses recycled waters. This coupling may ensure the elimination of the toxic compounds diluted in those effluents, mainly pesticides and their metabolites, no matter their salinity content. In fact this study will include photo-Fenton performance at extreme conditions in order to check if the recycled effluent could be submitted to a reverse osmosis process letting the brine, highly concentrated in salts, to be treated afterwards by the named AOP while the permeate is directly resent to the greenhouse cycle.

For the achievement of this general goal, specific objectives were established.

- Determining of photo-Fenton as the most convenient AOP to use in the frame of the pesticide decontamination

  For this purpose a comparison between photo-Fenton reaction and ozonation process performance will be set.

- Increase of the knowledge concerning different features of photo-Fenton reaction performance:
  - Optimization of working conditions
  - Endogenous interferences

  Experimental design and its analytical tools will be used to study photo-Fenton reaction. They will allow to optimize working conditions for different reaction media.

- Determining the effect of interferences by salinity content.

  Complex matrices containing target pesticides and salts will be submitted to photo-Fenton reaction and its performance will be monitored and compared.

- Asses the feasibility of using solar photo-Fenton with these particular effluent

Different matrices will be treated on solar photo-Fenton devices and their performance will be monitored and compared with artificially irradiated experiments.

- Testing the biocompatibility of resultant effluents and the real potential of the integrated system.

  Sequencing batch reactors will be set, loaded with photo-Fenton pretreated effluents, both those prepared with Milli-Q water and spiked with one pesticide, and those with a mixture of target substances and high salinity.

- Assessment of the suitability of photo-Fenton reaction/Slow Sand Filtration coupling.

  For this purpose, a lab scale Filtration Columns will be loaded with pretreated effluents and its performance will be monitored.

Three structurally different pesticides, from those most used in CENIT-MEDIODIA demo-greenhouses, will be the target compounds to simulate pesticide pollution, methomyl, imidacloprid and fosetyl-Al. Their commercial formulations will be used instead of pure compounds in order to study a closer to reality scenario, where inert ingredients could exert certain influence in different aspects of the systems that has to be taken into account.

In parallel, this work also aims to explore the potential of MBTs as new tools to apply on water treatment engineering. The main objective regarding these techniques was to characterize the biomass developed in the assessed bioreactors and try to establish a certain relationship between identified populations and the characteristics of the load. For this purpose cloning and sequencing were used to characterize the microbial community working on different bioreactors related to the coupling feasibility. Regarding to this techniques the following specific objectives were set.

- Determine the appropriate primers to carry out the sequencing process.

- Adaptation of the PCR protocol in order to obtain optimum replication results despite the salinity of the samples.

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EXPERIMENTAL METHODS AND MATERIALS

3.1. ANALYTICAL METHODS

3.1.1. DETERMINATION OF TOTAL ORGANIC CARBON (TOC)

TOC determination was performed with a Shimatdzu TOC-VCSN TOC analyzer combustion at 680ºC according to Standard Method 5220D procedures (APHA 1995). Potassium phthalate solution was used as calibration standard. Samples of 12 mL were filtered (0,45µm) previous to being injected in the device as a common practice for regular use of the analyzer. According to this, in this work, TOC identifies Dissolved Organic Carbon (DOC). For its part, dissolved inorganic carbon (CO_2 and carbonates) was removed by acidifying the samples with 1mL off 30% H_2SO_4 and bubbling synthetic air for 15 seconds also previous to injection. Total Nitrogen (TN) content of the samples was also measured by means of the specific module TNM-1 (Shimadzu) coupled to the installation.

3.1.2. DETERMINATION OF 5 DAYS BIOCHEMICAL OXYGEN DEMAND (BOD_5):

One of the tests performed to quantify the biodegradability of the samples was the Biological Oxygen Demand essay at 5 days (BOD_5), using Oxitop® (WTW Chemical) manometric bottles, following the procedures described in Standard Methods 5210D for respirometry analysis (APHA 1995). This method measured the consumed oxygen by the microorganism inoculum per water volume unit during 5 days of incubation at 20ºC. The method is based on the pressure change inside the bottle due to O_2 consumption and generated CO_2 capture by means of NaOH pearls allocated in a separated compartment.

Depending on the expected value of BOD, a different initial volume of target sample has to be used. In the case of lowest values, $BOD_5 < 40$ $mgO_2.L^{-1}$, 425mL of target solution was used for the analysis. It was buffered at pH 7.2 with 2.6 mL of NaH2PO4 1.5N and 0.865 mL of NH_4Cl, $MgSO_4$, $FeCl_3$, $CaCl_2$ and KOH were added as nutrients for the bacteria (corresponding concentrations for these solutions can be found in (APHA 1995)). The inoculum for these tests consisted on lyophilized microorganisms contained in so-called BOD-seed capsules supplied by Cole-Palmer. This inoculum was

revitalized suspended in pH 7 water and aerated during 2 hours, together with some of the named nutrients. 0.650 mL of the supernatant (the capsule contains solid coadjutants) of this suspension were introduced in each bottle. To prevent O_2 consumption by nitrification, this process was suppressed by adding 0.05 mL of 5 gL^{-1} N-***allylthiourea*** (***WTW*** Chemical. Solution No.NTH 600). The bottles were finally stored for 5 days at 20 ºC and the oxygen consumption was automatically calculated through the manometer's pressure drop.

3.1.3. DETERMINATION OF CHEMICAL OXYGEN DEMAND (COD)

Chemical Oxygen Demand (COD) test gives an indirect measure of the organic compounds contained in a water sample. This test is also performed for the quantification of biodegradability of a solution. The analysis determines the amount of oxygen required to totally oxidize the content of target sample by means of strong oxidant agents. However also oxidizable inorganic substances can be detected with the test and mask the results. Their presence in the samples should be avoided or at least taken into account.

COD analyses, carried out following the Standard Method 5220 D (APHA 1995), consisted in digesting 2.5 mL of the target solution with 1.5 mL of 0.2 N potassium dichromate solution and 3.5 mL of Ag_2SO_4/H_2SO_4 5.4:1000 g/g catalytic solution, for 2 hours in a digester, at a constant temperature of 150ºC. According to the Standard Methods 5220D, the theoretical occurring reaction for a typical organic molecule is reaction 32.

$$C_nH_aO_bN_c + dCr_2O_7{}^{2-} + (8d + c)H^+ \rightarrow nCO_2 + \frac{1}{2}(a + 8d - 3c)H_2O + cNH_4{}^+ + cCr^{3+} \quad \text{r. 1}$$

where $d = \frac{1}{6}(4n + a - 2b - 3c)$ eq. 1

The color change from the reduction of Cr^{7+} to Cr^{3+} allows obtaining absorbance measures for different samples by means of a spectrophotometer Odyssey DR/2500, working at 600 nm. To convert those measures into oxygen demand values calibration is needed. This calibration is based on potassium hydrogen phthalate ($C_8H_4O_4HK$) as primary standard. Phthalate solutions COD value can be obtained theoretically according to reactions 33 and 34.

$$41H_2SO_4 + 10K_2Cr_2O_7 + 2KC_8H_5O_4 \rightarrow 10Cr_2(SO_4)_3 + 11K_2SO_4 + 16CO_2 + 46H_2 \quad \text{r. 2}$$

$$2KC_8H_5O_4 + 15O_2 + H_2SO_4 \rightarrow 10CO_2 + 6H_2O + K_2SO_4 \quad \text{r. 3}$$

3.1.4. **Biodegradability Index BOD5/COD**

Among the different existing parameters to study the biodegradability of certain solutions, the ratio between the Biochemical Oxygen Demand at 5 days and the Chemical Oxygen Demand is considered a consolidated index for many investigators ((Burton and Tchobanoglous 1991; Chamarro et al. 2001; González et al. 2007). The BOD_5/COD ratio determines the amount of organic matter capable of being readily decomposed by means of biological degradation among the total amount of oxygen required to mineralize the whole content of organic matter into water and CO_2. Usually, COD values are higher than BOD_5 because many compounds that could be biorecalcitrant are easily oxidized under the strong oxidative conditions of the potassium dichromate involved in the Standard Methods COD measurements (Oppenländer 2002).

According to (Burton and Tchobanoglous 1991) this ratio also provides helpful information for water monitoring involved in self-purification processes, for example in cases where wastewater is released to the environment. Depending on its ration, the effluents can be classified according to the following table 1.

Table 1. Wastewater properties according to BOD_5/COD values

BOD_5/COD	**Effluent properties**
<0.1	Persistent organic pollutant, non-biodegradable
0.1 to 0.59	Incompletebiodegradation
>0.59	Easily and entirely biodegradable organic content

3.1.5. TOXICITY ANALYSIS

The biological response induced in different living organisms challenged by a chemical substance is diverse and depends on their sensitivity to toxicants. Bioassays can provide a measure of the whole-effluent toxic impact, a complex mixture of chemicals integrating different factors, such as pH, solubility, antagonism or synergism, bioavailability, etc. The use of a battery of bioassays involving different bioindicator species at different trophic levels is an efficient and essential tool for predicting environmental hazards to the aquatic ecosystem (Farré and Barceló 2003).

In the case of this work, acute toxicity tests were performed by means of an analyzer *Microtox ® M500*, according to the protocols developed by the supplier (Azur Environmental, Delaware, USA). In this test, luminescent *vibrio fischeri* bacteria (NRRL No. B-11177) were put in contact with solutions of the sample to study (45%, 22.5%, 11.25%, 5.62%) prepared with a solvent from the same supplier.Light production in these organisms is directly proportional to the metabolic activity of the bacterial population and any inhibition of enzymatic activity causes a corresponding decrease in bioluminescence (Farré and Barceló 2003; Parvez et al. 2006). According to this, the diminishment of luminescence for each case, after 15 minutes of contact,

compared to control solution which has not be in contact with the sample, gives an idea of the toxic effect of the analyzed sample.

Results obtained in this test can be expressed as $EC_{50,15min}$. It represents the percentage (% v/v) of the initial solution that causes a 50% of bioluminescence reduction in 15 minutes of contact. It can also be expressed as the inverse of this percentage in the shape of Toxicity Units, $TU=100/EC_{50}$.

3.1.6. ION-EXCHANGE CHROMATOGRAPHY

Inorganic anions and cations were detected by means of ion-exchange chromatography. It is a well-known chromatographic process that allows the separation of ions and polar molecules based on their charge and their consequent coulombian interaction with the stationary phase. The liquid sample is injected in the device and passes through a column where the stationary phase retains the electrolytes more or less intensely depending on the charge and affinity (for cations analyses, the column filling releases positively charged species from its constituents, while anionic columns release negatively charged molecules to balance the charge of the retained anions). Specially designed eluents for cation and anion determination are able to untie the attached electrolytes. They make them through the exit of the column ordered according to their retention time which, all at once, depends on the affinity of the charged specie with column filing.

The device used for cations such as ammonia detection was a Dionex DX-120 ion chromatographer equipped with a 4 mm × 250 mm Dionex Ionpac CS12A column. Anions such as nitrates and phosphates were determined with a Dionex DX-600 ion chromatograph and 4 mm × 250 mm Dionex Ionpac AS11-HC column.

3.1.7. DETERMINATION OF PESTICIDE CONCENTRATION

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Pesticides depletion along each experiment was monitored by a high performance liquid chromatography with photodiode array detector that consisted on:

- Waters Degasser
- Pumping system Controller Waters 600
- Autosampler Waters 717
- Columns oven and temperature controller
- Photodiode array detector Waters 996
- Millenium Software 2005 and Empower Software 2007

The column used was a Mediterranean Sea18, 5µm 25x0.46cm (Teknokroma). The mobile phase, composed by a mixture of water and acetonitrile (both from Merck) (60:40), was delivered at a flow rate of $1mL \cdot min^{-1}$. In the cases with higher salinity content, a peak related to NaCl masked methomyl peak, so another method was used

with a mobile phase composed by 2.5mL of 80% H_3PO_4, 25mL of methanol, diluted to 500mL with milli-Q water. In that case the flow was 0.7mL·min^{-1}.

The concentration of the two pesticides could be measured in the same analysis thanks to the ability of the software to extract from the resulting spectrum the wavelengths corresponding to the highest UV absorbance for both pesticides: 232.5 nm in the case of methomyl and 269 nm in the case of imidacloprid.

TANDEM SPECTROSCOPY

Because of its chemical characteristics (low molecularmass, ionic structure, and lack of UV absorption or fluorescence) fosetyl-Al is rather difficult to determine by conventional (reversed-phase) liquid chromatography (LC). One alternative to this technique is liquid chromatography with electrospray tandem mass spectrometry (LC/MS/MS). It combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. After passing through the column, the components of the sample are ionized. The ions are separated according to their mass-to-charge ratio by electromagnetic fields. Then the ions are detected by an analyzer and their signals are processed into mass spectra.

In the particular case of the high polarity of the alkyl phosphates, it required the use of an ion-pairing reagent to obtain sufficient retention time in the chromatographic step. It allows the separation of highly polar interferences (salts) and analytes. Thus, a volatile ion-pairing reagent, tetrabutylammonium (TBA) acetate, was selected to prevent interface blockage(Hernández et al. 2003).

3.1.8. TOTAL SUSPENDED SOLIDS AND TOTAL VOLATILE SUSPENDED SOLIDS

Total suspended solids, TSS, and Total volatile suspended solids, TVSS, can be used to give an idea of the evolution of the biomass in biological reactors. To perform these tests, 0.45 μm pore size nitrocellulose filters were previously pretreated using them to filter 500 mL of pure water and being dried for 2 h, at 105ºC, and calcined for 15 min, at 550ºC, according to Standard Methods 2540D and 2540E, respectively. Once the filters are ready, their weigh is recorded as W_1 (mg). Then know volumes of sample, V (L), were passed through filters, which were subsequently dried at 105ºC for 1 h (2 replicates of each samples should be done, using two different filters for two identical volumes of the same sample). After this period filter weigh was again recorded as W_2. Finally the filters were calcined at 550ºC for 15 min, expecting all the organic matter to volatilize. The final filter weigh was also recorded as W_3.

Total suspended solids could be calculated through de difference between W_1 and W_2, it represents the total quantity of solids retained by the filter once the water is evaporated; total volatile suspended solids is related to the difference between W_1 and W_3, it constitutes the quantity of solids retained by the filter that are volatile at temperatures

under 550ºC, what comprises big majority of organic substances, associated with the biomass content. Eq. 2 and 3 allow calculating these parameters.

$TSS{=}W1{-}W2TSS = \frac{W_1 - W_2}{V}$ (mg·L^{-1}) eq. 2

$TVSS = \frac{W_1 - W_3}{V}$ (mg·L^{-1}) eq. 3

3.1.9. BATCH REACTORS TEST

The SBRs are activated sludge systems running on the fill and draw principle. In the SBR operation, the reaction and sedimentation processes are carried out sequentially in the same tank rather than simultaneously in separate tanks as for the conventional activated sludge system (Chan and Lim 2007). The operation of the SBR system is commonly carried out sequentially in four steps, fill, react, settle and draw, respectively, as can be seen in fig. 23. This simple setting of biological reactor is used to get an idea of the biodegradable character of a sample (Bacardit et al. 2010), to complement the information given by BOD_5/COD ratio, toxicity essays, etc. (González 2009). This system allows to:

- Get reliable data about the different organic matter fractions present in the effluents depending on the initial conditions of the samples from the point of view of biodegradability:
    - Rapidly biodegradable
    - Biodegradable
    - Biorecalcitrant

- Observe if the conventional sludge suffers any kind of toxicity or inhibition from the potential biorecalcitrant content of the sample.

- In this work, it also allows to quantify the persistence of different concentrations of pesticides and the inert ingredients of the commercial formulations, in contact with conventional non acclimated sludge.

- Observe acclimation effects of different substrates.

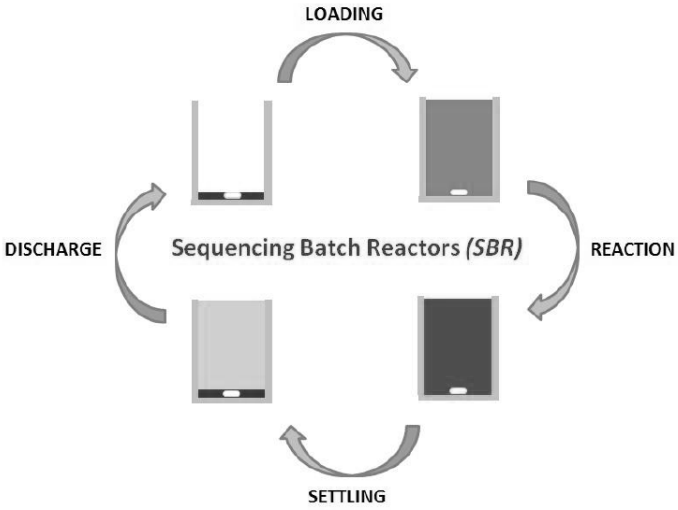

Figure 1: Operation stages in a SBR (Esplugas 2010)

During the *loading* step, the target solution is introduced into de reactor, where it is mixed with activated sludge. The biomass is suspended together with the target solution and bacterial degradation takes places under aerobic conditions. Then, the *reaction* between the biomass and the target compounds are takes place in the vessel. Once the reaction is completed (the monitored parameters such as dissolved oxygen, TOC, pesticide concentration, etc., have reached constant values), mixing is stopped and a *settling* stage takes place in order to separate the biomass from the treated solution. The supernatant is then *discharged*, leaving the reactor only with the activated sludge, back to its initial position and ready to be loaded again.

In the experiments performed in this work, SBR's consisted in 1L Erlenmeyer flasks continuously stirred and aerated to maintain aerobic conditions until the settling stage starts. The experimental set up is depicted in fig. 24.

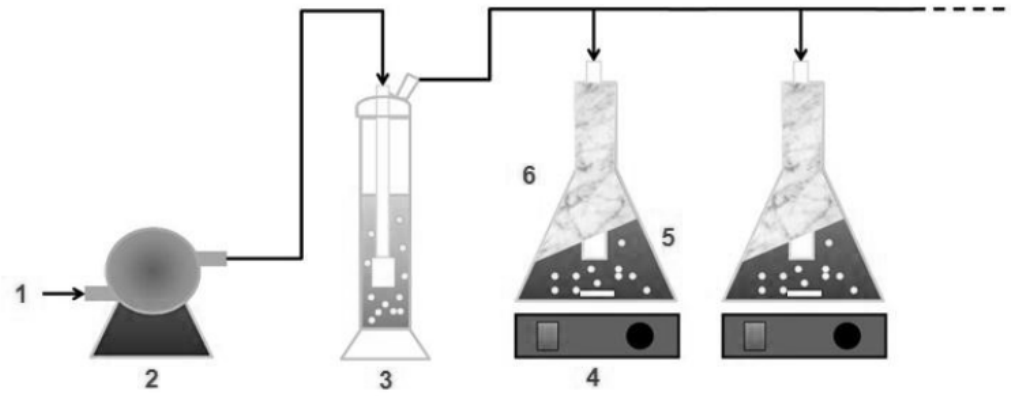

Figure 2: Sequencing batch reactors set up (Esplugas 2010); (1) air inlet, (2) air pump, (3) humidifier, (4) magnetic stirrer, (5) reactor, (6) alumina foil.

In this installation, oxygen was supplied in excess by means of a Rene Air 200® air pump with ceramic diffusers. This air was humidified prior of being introduced into the SBR to avoid water evaporation in the vessels. The performance of the reactors was

carried out at room temperature, always in the range 20 and 25 ºC. Each reactor was covered with aluminum foil to avoid the proliferation of algae inside the reactor as a consequence of solar light incidence.

All the essayed samples needed to be adapted prior to be introduced in the reactor. With this purpose, directions from the Respirometric Standard Method 5210 D (APHA 1995)were followed. According to them, the solutions were set at room temperature, and the pH was raised to 7 by the addition of NaOH. Then, these solutions were buffered with 6 $mL.L^{-1}$ of NaH_2PO_4/Na_2HPO_4 1.5 N, and 2 $mL.L^{-1}$ of each of the different nutrients solutions were added: $CaCl_2$, 0.25 N; $MgSO_4$ 0.41 N; NH_4Cl 0.16 N and trace elements solution (consisted of traces of $MnSO_4$, H_3BO_3, $ZnSO_4$, $(NH_4)_6Mo_7O_{24}$ and $FeCl_3$-EDTA). The addition of $FeCl_3$ was suppressed in the photo-Fenton effluents given that iron ions were already present in the solution. In the control reactors, the same iron was added as the initial quantity photo-Fenton reaction had.

3.2. EXPERIMENTAL DEVICES

3.2.1. ARTIFICIAL LIGHT PHOTO-FENTON REACTOR

Photo-Fenton reaction was carried out in a 2L Pyrex jacketed stirred vessel (inner diameter 11cm, height 23cm), with three 8W (nominal power) black light lamps (Philips TL 8W-08 FAM, 30 cm length), axially arranged to the reactor. The emission spectrum of these lamps goes from 350 to 500 nm, although the maximum emission is located at 365 nm. Depending on the "age" of the lamps, the photon flow arriving to the system (measured by periodic actinometries) changed from 7.10 to 5.56 μEinstein s^{-1}.

Fig. 25 is a schematic design of the device. As can be seen it is connected to a thermostatic bath that lets the experiments evolve at controlled temperature of 21ºC. The vessel is covered with aluminum foil to prevent personal damage. pH was set to 2.7 at the beginning of the reaction with H_2SO_4 (Panreac) diluted solution.

The reaction solution was prepared in 2L flask with deionized water, the proper amount of pesticides and salts if necessary, depending on the experiment, was added (excess volume was prepared if any previous analyses were needed). The pH was adjusted to 2.7 (optimum for the equilibrium $[Fe^{2+}]/[Fe^{3+}]$ to achieve its highest ratio) with H_2SO_4 aqueous solution. Then, powder $FeSO_4 \cdot 7H_2O$ (Panreac PA) as needed was included in the solution as the source of Fe^{2+}, and pH was readjusted if required. The flask was poured inside the reactor and after 2 min of preheating, the lamps switched off are introduced in the media attached to the lid of the reactor. The device was properly closed and secured, and the necessary amount of H_2O_2 (Panreac PRS, 30%, w/v) was added under vigorous magnetic stirring. In that very moment, the UV lamps were switched on simultaneuously and the reaction time started. In most of the experiments, the reaction was let evolve until the total consumption of hydrogen peroxide.

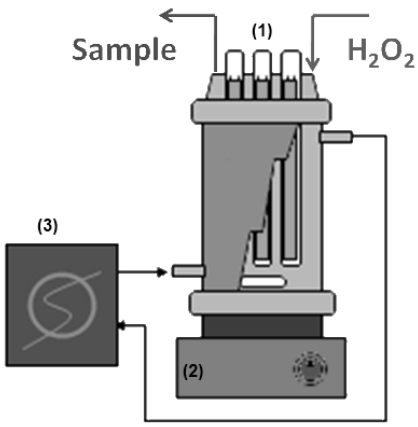

Figure 3. Photo-reactor scheme. (1) UV lamps, (2) Magnetic stirrer, (3) Thermostatic bath.

Samples were withdrawn periodically from the reactor to monitor H_2O_2 consumption, dissolved Fe^{2+} presence, concentration of pesticide, toxicity, COD, and remaining TOC. Depending on the intentions of the experiments, the concentration of remaining H_2O_2 was monitored using Quantofix® test sticks (Macherey-Nagel) or by the metavanadate method, based on the reaction of H_2O_2 with ammonium metavanadate in acidic medium, which results in the formation of a red-orange color peroxovanadium cation, with maximum absorbance at 450 nm (Nogueira et al. 2005; Souza et al. 2013). Meanwhile, iron concentration was determined by colorimetry with 1,10-phenantroline according to ISO 6332(Internacional Organization for Standarization 1982). For the rest of analyses, performed with the different chromatographic systems described above and the Shimadzu analyzer, the reaction need to be previously quenched with $NaHSO_3$, 40% w/v (Panreac), consuming residual H_2O_2 in the withdrawn samples. In the case of toxicity analyses and COD tests, the sample was quenched with a solution with a known concentration of catalase (Sigma Aldrich), which contribution on COD values can be predicted and subtracted, and no toxicity can be detected in the microtox analysis.

The resulting effluent as kept to carry out COD and BOD analyses in some cases, in others, to feed the slow sand filtration column described below or the above depicted sequencing batch reactors.

Special particularities on the reactor performance and the analyses performed are consigned in the respective Appendices.

3.2.2. SOLAR LIGHT PHOTO-FENTON REACTOR

Solar photo-Fenton, the experiments were performed in two different locations.

The bigger photo-reactor was sited in Tabernas, Almería, at Plataforma Solar de Almería (PSA), 37º 05' 49.50" N 2º 21' 53.98" O. The experiments were carried out under sunlight in compound parabolic collectors (CPC) at the PSA. A complete description of the pilot plant can be found at (Blanco et al. 2000; Kositzi et al. 2004). It is made up of twin systems, each having three collectors, one tank and one pump. Each collector (1.03 m^2 each) consists of eight Pyrex tubes connected in series and

mounted on a fixed platform tilted 37° (local latitude). The schematic configuration of the system is shown in fig. 26.

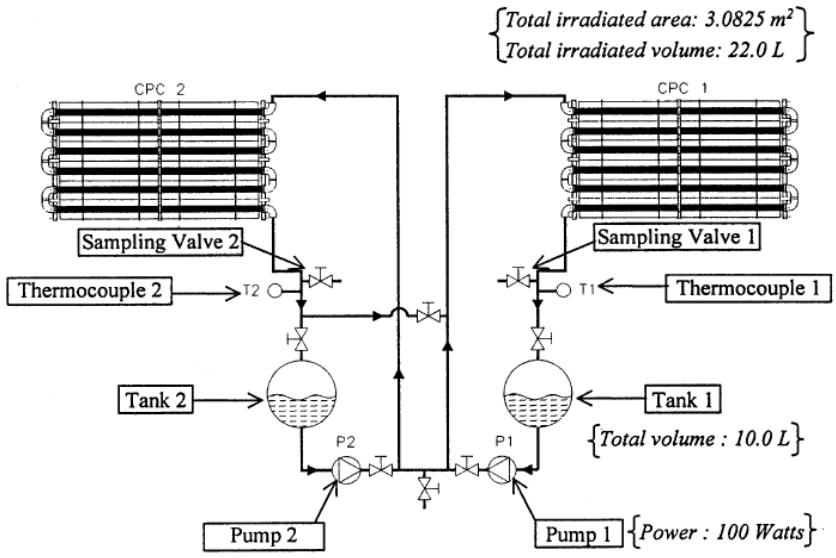

Figure 4. Schematic representation of the solar photocatalytic pilot plant reactors (two cpcmodules+tank+pump+connections) (Kositzi 2003).

The water flows at 20 L min^{-1} directly from one module to another and finally into a tank. The total volume of the reactor (35 L) is separated in two parts: 22 L total irradiated volume, and 13 L the dead reactor volume (tank + connecting tubes). At the beginning of the experiments, with the collectors covered, all the chemicals are added to the tank and mixed until constant concentration is achieved throughout the system. H_2O_2 is added and homogenized. Then the cover is removed and samples are collected at predetermined times. As the CPCs do not concentrate light inside the reservoir but just in the Pyrex tubes, the system is outdoors and is not thermally insulated, the maximum temperature achieved inside the reactor during the experiments is 35°C. Solar ultraviolet radiation UV was measured by a global UV radiometer (KIPP & ZONEN, model CUV3), mounted on a platform tilted 37° (the same angle as the CPCs). It gives an idea of the energy reaching any surface in the same position with regard to the sun.

The smaller device consisted on a verification handmade scale plant located in the University of Barcelona (latitude 41º28'N, longitude 2º06'E, sea level), between August and September 2010. As can be seen in fig. 27, the photo-reactor comprises a module of 6 parallel CPCs (theoretical concentration factor of 1, C_{CPC}=1) made of polished aluminum, with a total mirror´s area of solar irradiation caption-reflection of 0.228 m^2, tubular quartz receivers, attached to a galvanized surface angled 41º (local latitude). The irradiated volume was 0.95 L from a total reaction volume of 5 L allocated in a mechanically stirred reservoir tank of 10 L. The solution was continuously recirculated through the photo-reactor piping and the reservoir tank, employing a peristaltic pump (Ecoline vc-380, Ismatec). Temperature was monitored. During solar experiments, irradiance was always followed using a radiometer (Delta OHM, LP 471 UVA). It was placed on the CPCs platform with the same orientation and inclination. It provided data in terms of direct irradiation (315-400 nm) in Wm^{-2}.

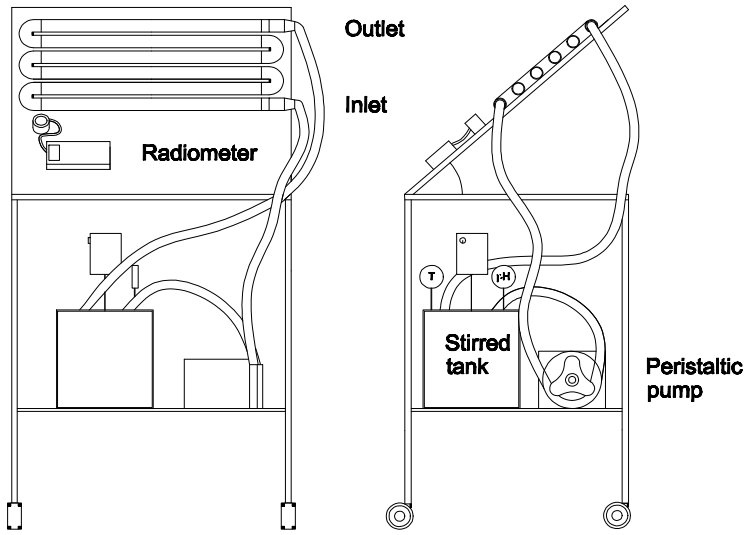

Figure 5.Schematic representation of the solar photocatalytic lab scale reactor.

3.2.3. OZONATION DEVICE

As can be seen in fig. 28, ozonation experiments were carried out in a 1.2 L cylindrical glass vessel with a thermostatic jacket connected to a bath that kept constant temperature during the experiments, fig. 28. Back on track, in each ozonation experiment 1 L of the different effluents to treat was loaded. Ozone was generated in a Sander Labor Ozonizator® that used 1 bar pure oxygen (Air Liquide®) as inlet gas, which could produce ozone at rates from 0-30 $g \cdot h^{-1}$by means ofcorona discharge method. This process requires the ozonator to be also constantly refrigerated by circulating tap water through its cooling system to avoid overheat caused by the continuous electric discharges produced inside the device required to form the O_3 molecule.

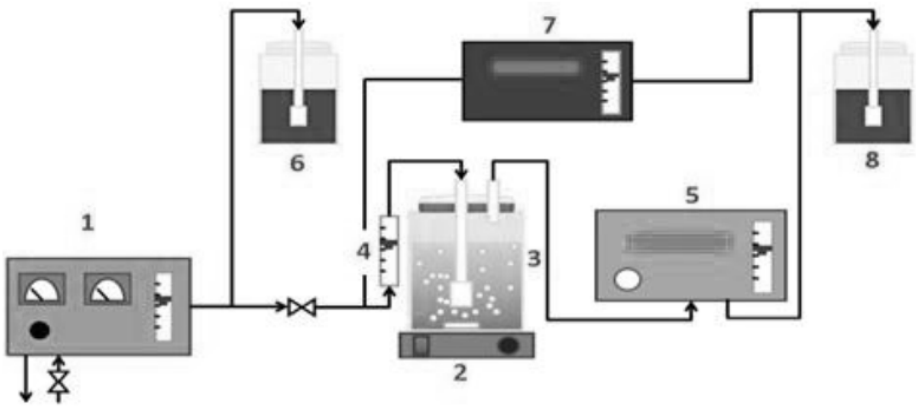

Figure 6.Ozone installation (Esplugas 2010). (1) Ozonator, (2) stirrer, (3) reactor, (4) ozone flow controller, (5) outlet ozone detector, (6) KI solution, (7) inlet ozone detector, (8) KI solution.

The generated ozone was bubbled inside the reactor through inert metallic gas diffusers. The reactor was continuously stirred by means of a magnetic stirrer to ensure homogeneity during the tests and provide a better contact between the liquid and the gas phase. Ozone input and the output were monitored using an ozone gas phase analyzer BNT 963 (range 0-200 g O_3/m^3). Furthermore, the outlet gas from the reactor was sent to a saturated potassium iodide solution to transform the unreacted ozone back into oxygen by means of reaction 35.

r. 4

The whole set of ozone experiments were performed inside a fume hood to ensure a secure operation in case that an unexpected ozone gas release could occur.

3.2.4. SLOW SAND FILTRATION COLUMN

A scheme of the slow sand filtration column is depicted in fig. 29. Filtration device consisted of a glass cylindrical reactor of 110 cm height, 5 cm diameter, covered with aluminum foil to avoid light penetration and the development of photosynthetic algae, filled (up to 101.5 cm) with expanded clay particles (2.5-5.0 mm), and loaded constantly, with an average down flow of 0.3 $mL \cdot min^{-1}$, supplied by a peristaltic pump, from a stirred tank. This reservoir tank is continuously aerated ensuring oxygen saturation. The empty volume not occupied by clay was calculated approximately as 850 mL, the elapsed time for the effluent front to traverse the column length was about 46h. The SSF was continuously operated at a filtration rate of 0.01 $m \cdot h^{-1}$.

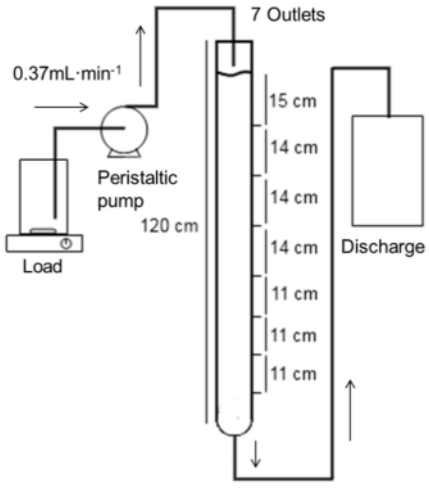

Figure 7.Slow sand filtration scheme.

The SSF column device used for this work consisted on a glass cylinder ($Ø_{inside}$=5.4 cm), filled with 1m of 0.1-0.4cm Filtralite particles (Saint-Gobain Weber, France). It counts with 7 different outlets where liquid and solid samples can be withdrawn. The first one is 20 cm below the top of the column, but 15cm below the surface of the

packing. The last one is 2 cm above the mesh filter that retains the expanded clay grains. Samples are regularly withdrawn from this last outlet except when it is done from the all seven outlets to check the state of the process all over the column. The device is fed by means of a peristaltic pump, working 0.37mL·min^{-1}, HRT of 4.3 m·d^{-1} that impulses the effluent from a continuously stirred tank to the top of the column. The study of the column performance was carried out feeding it with replicates of previously photo-Fenton treated effluents for a certain amount of weeks.

3.3. MOLECULAR BIOLOGY TECHNIQUES AND PROCEDURES

Molecular biology techniques employed in this thesis are based on cloning and sequencing of the DNA fragment, or gene, that codes 16S rRNA, in order to identify bacterial populations and create what is called 16S rRNA libraries. This methodology implied different steps that comprise the nucleic acids extraction, amplification and cloning of the named fragment, followed by sequencing and finally identification of the isolated clones by using phylogenetic databases.

3.3.1. 16S RRNA

16S ribosomal RNA (or 16S rRNA) is a component of 1.542kb (kilo base pairs, 10^3 base pairs) of the small subunit (SSU[1]) of prokaryotic ribosomes, found in all bacteria and archaea.

[1] Together with the SSU, the large subunit, LSU, sandwich the mRNA as it feeds through the ribosome for translation into proteins (Andersen et al. 2011).

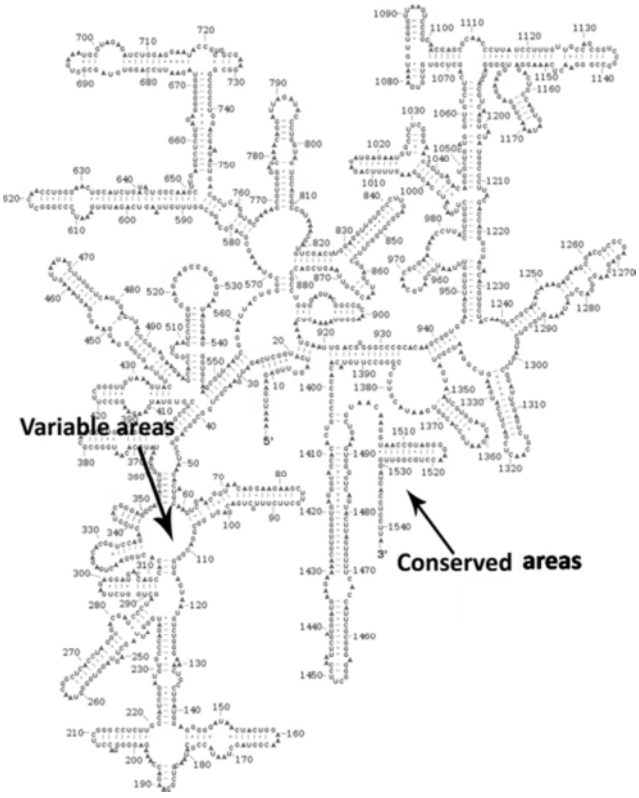

Figure 8. 2D representation of E. coli 16S rRNa. *Note: It is not this "molecule" the one sequenced, but the DNA fragment that code its production.*

The 16S rRNA gene is a commonly used tool for identifying bacteria. First of all, it allows differentiate prokaryotes from eukaryotes. While there is a homologous gene in eukaryotes, the 18S rRNA gene, it is distinct, thereby rendering the 16S rRNA gene a useful tool for extracting and identifying bacteria as separate from plant, animal, fungal, and protist DNA within the same sample. Sencondly, 16S rRNA can be considered an evolutionary clock that enables discriminate different genotypes separated on the evolution tree. Ribosomes (and correspondingly the DNA that codes for them) have been mostly conserved over time, meaning that their structure has changed very little over time due to their important function, translating mRNA into proteins. But even within this gene there are parts that have been conserved along the eras, while some others are highly variable. This is due to the structure of the ribosome itself. With the way the ribosome folds, it creates bonds in some places (conserved regions) while other portions are looped and unbounded (hypervariable regions), fig. 30. In those regions is where the differentiation between species or families, etc. is expressed, providing genotyping-specific signature (Andersen et al. 2011).

3.3.2. DNA EXTRACTION

This first step consists on the isolation of nucleic acids from the sample biomass and letting it ready for following downstream processing.

DNA extraction comprises some commons steps that traditionally were performed separately and manually. Nowadays there are in the market plenty of commercial kits adapted to different sources' samples that facilitates de process, enables its automation and eliminates contamination that can interfere in downstream processing. The main steps can be summarized as follows:

- **Cell lysis or disruption**: the cell membranes are broken by means of anionic surfactants (SDS is commonly used), which can break down fatty acids and lipids associated with the cell membrane of several organisms. This chemical process could be combined, especially in the case of seawater samples (with stronger membrane walls), with physical processes such as blending, grinding or sonicating.

- **DNA cleaning**: in cell disruption, all cell materials are released to the reaction media. Including all the different types of nucleic acid based molecules, DNA, RNA, proteins, etc. To get rid of non DNA-species that will interfere in downstream processing, enzymes such as protease and ribonucleases, RNase, are added in this step. In order to protect DNA from degradation, some protocols also include the addition of chelating agents to scavenge divalent cations such as Mg^{2+} and Ca^{2+} that inactivates desoxiribonucleases. Applications such as soil or sewage waters samples sometimes require additional cleaning products in order to remove substances that could inhibit PCR, such as humic acids, phenol, isopropanol, etc. The final step of the cleaning process always include one or several centrifuge cycles in order to remove the impurities and debris and release diluted DNA free of contamination.

- **DNA binding**: consist on a separation step in which DNA is linked to a solid substrate in order to perform the cleaning of its surrounding liquid media, replacing it by a sterile fluid media in the resolubilization step. Usually, for this step, the sample coming from cleaning is mixed with a salt solution and it is made to pass through silica filters. The high conductivity of the solution causes DNA to bind to the filter, while other non-DNA, organic and inorganic material flows across it. Ethanol washing solutions may contribute to remove that contamination forcing it to leave the filter, while the insolubility of DNA in this alcohol increases its binding to the silica mesh.

- **DNA resolubilization**: once every remaining ethanol is eliminated (ethanol can also inhibit PCR), sterile alkaline buffer, or even ultra-pure water, selectively desorbs and solubilize DNA. Once this step is completed, resulting extracted DNA could be stored at -20ºC for long periods.

In this thesis, Powersoil DNA Isolation kit (MOBio Laboratories Inc.) was used to extract DNA samples from different biological reactor devices and its protocol was carefully followed.

3.3.3. PRELIMINARY QUANTIFICATION

A preliminary way to quantify DNA content and to know its purity (from contaminant such as proteins or other biopolymers) could be performed measuring the absorbance of the DNA solution at 260 and 280 nm with a traditional spectrophotometer. DNA absorbs light at 260 and 280 nm, while aromatic proteins absorb light at 280 nm; a pure sample of DNA has the 260/280 ratio around 1.8 if it is relatively free from protein contamination. As closer to this value the ratio of absorbance is, as pure the DNA extracted solution is.

This test could be performed right after DNA extraction, usually diluting between 1 to 5 µL of extraction result in 500 µL of ultra-pure water. If the results are not satisfactory further cleaning could be applied and the test could be done again expecting better values. In those cases when extra cleaning was needed; PowerClean® DNA Clean-Up Kit (MOBio Laboratories Inc.) was used.

3.3.4. GEL ELECTROPHORESIS

Gel electrophoresis analyses are conducted in two stages of the process. The first time, after preliminary quantification, it allows confirming the presence of DNA in the extraction resultant solution or after a further cleaning. The second time ensures the good quality of PCR products.

This procedure is based in the effect of a difference of electric potential between two extremes applied on an agarose gel immerse in an electrolyte solution. The negative charges of DNA strains, which have been loaded in the anodic extreme of the gel, forces the molecules to move from the anode to the cathode under the electrical field. Depending on the size of the strain the migration velocity will be different, so when the voltage ends the different size strains will have reached headed or backward positions. As can be expected, lighter strains (shorter in base pairs) will move forward than heavier strains. Fig. 31 represents the process.

a) b)

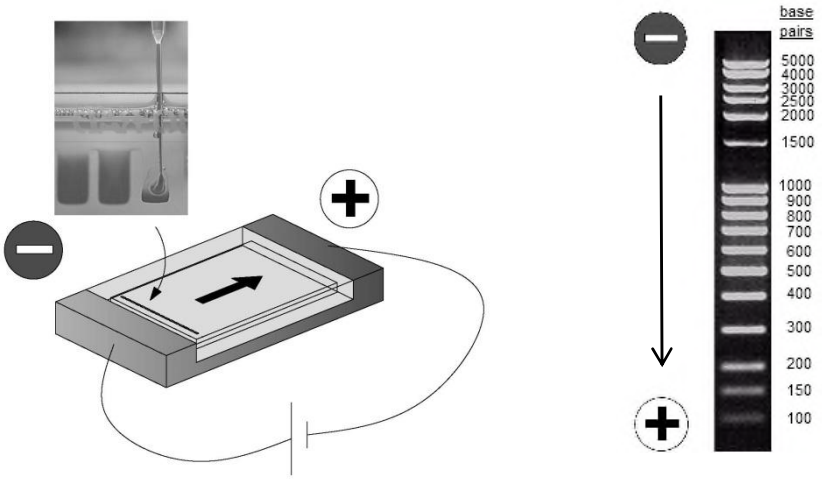

Figure 9. Gel electrophoresis. 31a. Schematic representation of the process. DNA samples are loaded close to the anode and the voltage generated by the power supply impulses them towards the cathode. 31b. Advance of the different DNA strands contained in the ladder mix depending on their sizes.

With the purpose of this test, a Gel Electrophoresis System AGT-3 (VWR) was connected to a VWR Power Supply Model 202 (VWR). Gels consisted in 100 mL of 1% agarose dissolved in 1xTAE (Tris-Acetate-EDTA) solution after its heating in a microwave. During the cooling of the agarose solution a gel stainer substance was added. This substance will bind to DNA and will glow under UV light excitation, denoting indeed the presence of DNA. In the first uses, the stain was Ethidium Bromide (Pronadisa) but its carcinogenic risks forced the change to a safer substance and SYBR® Safe DNA Gel Stain (Life Technologies) was used from then on. After staining and previously to complete cool the solution is poured into a mold where it will solidify in around 20 min. A special "comb" is placed in the mold previous to solidification in order to leave smalls wells in the gel were DNA samples will be loaded.

For runs with DNA extraction products, 1µL of 6X Loading buffer (Eppendorf) will be mixed with 5µL of different extracted DNA samples and added to each well. Meanwhile, for PCR product, the loading buffer will be combined with 2.5 µL of deionized water and 2.5 µL of PCR product. In both cases, together with the set of samples, 5 µL of DNA Ladder (AppliChem), a solution of DNA molecules of different lengths, in this case ranging from 100 to 5000 base pairs strains, is loaded to a spare well, Fig. 31b. It is used as a reference to estimate the size of unknown DNA molecules. In addition it can be used to approximate the mass of a band by comparing the intensity to bands in the ladder to the band of interest.

Every run consists in applying 120V constant voltage during 45 min by means of the Power Supply system, immersing the gel in 1X TAE as conductor solution. The resulting gels will be visualized in a UVIDOC gel documentation system (Uvitec) under blue-green UV-light (transilluminator), where pictures of them will be taken. Fig. 32 shows two examples of photographed gels.

a) b)

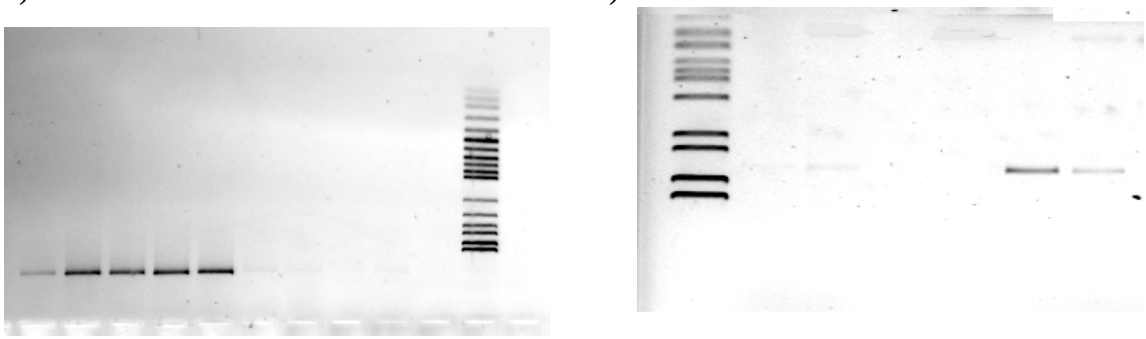

Figure 10. Gel electrophoresis examples. 10a. Extracted DNA fingerprints. 10b. PCR products fingerprints

Direct visualization of the gels by transiluminator devices shows them black with bright white signals for DNA. However it is a convention that the pictures of gels are always represented with inverted colors, in order to make its picturing and interpretation easier. In fig. 32a gel electrophoresis of different DNA extraction products is depicted. On the right side, with different bright bands in the same track there is the ladder, while the diffused signals correspond to the extracted DNA samples. The extraction product is composed by a bunch of DNA and protein strains with a myriad of sizes so the fingerprint in gel electrophoresis will always show a certain dispersed signal. In opposition to this, PCR products fingerprints are expected to be clearer, as can be seen in fig. 32b. Any diffuse signal around it could be interpreted as contamination and interference in the polymerase reaction and would obstruct the following processes. Depending on the primers used, the size of the PCR product will be different. In this case corresponds to a size of around 1480 bp due to the primers 8F, 1492R. The size of the ladder bands closer to the PCR products correspond to 1000 and 2000 bp (ladder used in this picture has 3000 bp as its heaviest band, not like in the one depicted in fig. 31b)

3.3.5. POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) is a molecular biology technique used to copy and amplify a single Deoxyribonucleic Acid (DNA) sequence into thousands or millions copies of a particular section of that sequence. Like gel electrophoresis, PCR is employed in two occasions in the whole process; once after DNA extraction, for increasing the concentration of extracted DNA, and once again for the sequencing. The general concept is common for both, but in this section the particularities of the first use will be explained, while sequencing PCR details will be described later.

PCR method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. In the case of 16S rRNA library method,

chosen primers correspond to sequences common for almost all the bacteria and are complementary to the extremes of the target sequence. Table 22 recaps a list of some of these universal primers, also known as oligonucleotides.

Table 2.Universal primers. F stands for forward while R stands for reverse. Shaded in grey, F and R employed for PCR in this thesis.

Primer name	Sequence (5'-3')[2]
8F	AGA GTT TGA TCC TGG CTC AG
U1492R	GGT TAC CTT GTT ACG ACT T
928F	TAA AAC TYA AAK GAA TTG ACG GG
336R	ACT GCT GCS YCC CGT AGG AGT CT
1100F	YAA CGA GCG CAA CCC
1100R	GGG TTG CGC TCG TTG
337F	GAC TCC TAC GGG AGG CWG CAG
907R	CCG TCA ATT CCT TTR AGT TT
785F	GGA TTA GAT ACC CTG GTA
805R	GAC TAC CAG GGT ATC TAA TC
533F	GTG CCA GCM GCC GCG GTA A
518R	GTA TTA CCG CGG CTG CTG G

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase, an enzyme originally isolated from the bacterium *Thermusaquaticus*. This DNA polymerase enzymatically assembles a new DNA strand from nucleotides (in the shape of deoxynucleoside triphosphates, dNTPs), by using single-stranded DNA as a template and DNA primers, which are required for initiation of DNA synthesis all immersed in a buffer solution. Two primers are used altogether in this kind of PCR (only one is used in each sequencing PCR), both are complementary to the 3' ends of each of the sense and anti-sense strand of the DNA target, as can be seen in fig. 33.

[2]Nomenclature 5'-3' is used to determine the direction of the strand when talking about DNA or RNA synthesis. Nucleotides are composed by a nitrogenous base, a pentose and a phosphorous group bonded to one of the carbons that compose the pentose. Number 5' is given to that carbon bonded to the phosphorous group, while 1' is the one bonded to the nitrogenous base. In turn, DNA and RNA strands are composed by nucleotides joined together by condensation. The phosphorous group of one nucleotide, once changed into a phosphate group, is joined to the 3' carbon of next nucleotide on the growing chain (Kratz 2009). According to this, primer 8F strand has on one of its ends the 5' carbon from adenosine and on the other, the 3' carbon of the guanine, ready of being condensate to a new nucleotide as part of the replicated sequence. Fig. 33 gives more information about this.

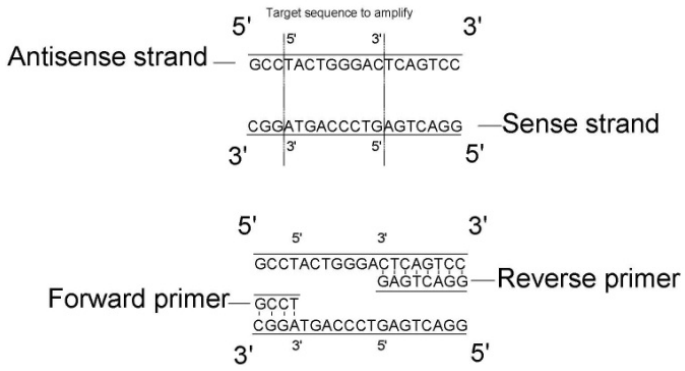

Figure 11. Schematic and simplified representation of how the primers bind to the strands to replicate

The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample to a defined series of temperature steps. These thermal cycling steps are necessary first to physically separate the two strands in a DNA double helix at a high temperature in a process called DNA melting. At a lower temperature, each strand is then used as the template in DNA synthesis by the DNA polymerase to selectively amplify the target DNA.

The PCR is commonly carried out in a reaction volume of 10–200 μL in small reaction tubes (0.2–0.5 mL volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use of the Peltier effect, which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current.

Typically, PCR comprises a series of 20-40 repeated temperature cycles, each consisting of 2-3 discrete temperature steps. The cycling is often preceded by a single temperature step (called hold) at a high temperature (>90°C), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the stability of the enzyme used for DNA synthesis, the concentration of dNTPs in the reaction, and the melting temperature[3], T_m, of the primers.

After an **initialization step** when the samples are heated to a temperature between 94 and 96 ºC, which is held for 1-9 minutes, a succession of several 3 steps cycles takes place, as it is represented in fig. 34.

- **Denaturation step:** This step is the first regular cycling event and consists of heating the reaction to 94–98 °C for 20–30 seconds. It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

- **Annealing step:** The reaction temperature is lowered to 50–65 °C for 20–40 seconds allowing bonding of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees Celsius below the T_m of the primers used. Stable DNA-DNA hydrogen bonds are only formed when

[3]At melting temperature DNA double helix dissociates into single strands.

the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA formation.

- **Extension/elongation step:** The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential (geometric) amplification of the specific DNA fragment.

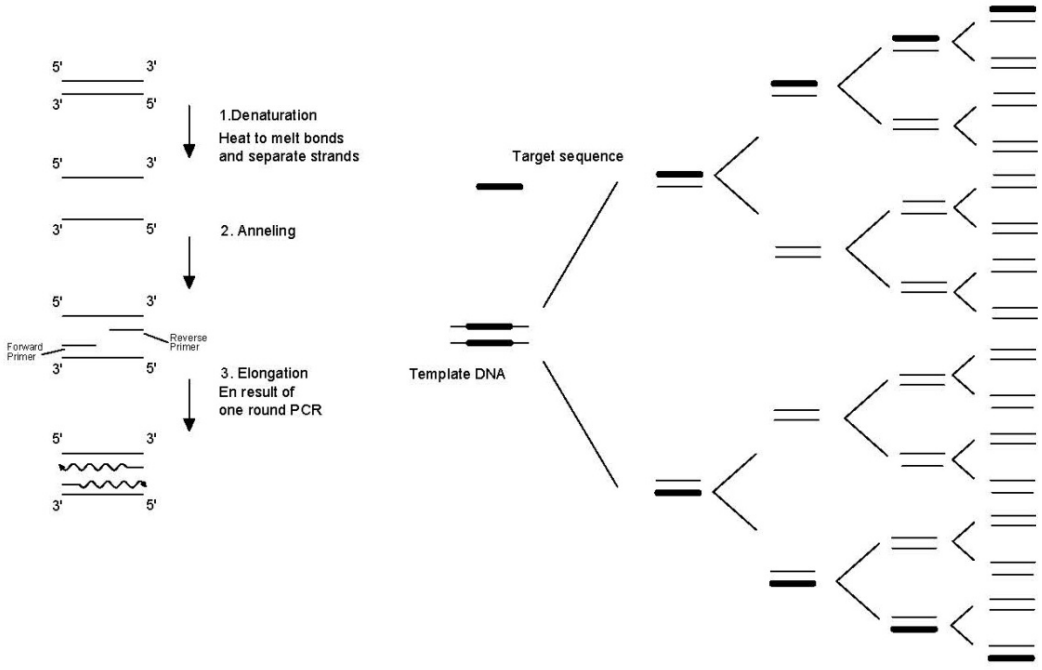

Figure 12. Schematic representation of the 3 basic steps of the PCR

After the desired number of cycles is completed, two extra steps take place, in order to stabilize the resulting PCR product:

- **Final elongation:** This single step is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

- **Final hold:** This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

Description of the cycle used in this thesis follows in table 23.

Table 3. PCR cycling description.

Step	T (ºC)	t (min)	
Preparation	from 20 to 95	2	
Denaturation	94	0.5	
Annealing	50	0.5	32 cycles
Extension	72	0.5	
Hold	68	10	
Conservation	4	∞	

PCR reactions were performed with Taq DNA Polymerase (Quiagen), with 8F and 1492R primers and according to the doses from table 24. ABI 9700 (Applied Biosystems) was the thermocycled device that worked on the conditions described in table 23.

Table4. PCR ingredients.

	v (µL)
Water	10.75
8F	0.25
1492R	0.25
TaqPolymerase	12.50
DNA template	1.25
Total volume	25.00

3.3.6. CLONING

Although numerous methods are now available for direct sequencing of PCR, cloning of amplified DNA for sequencing in vectors remains an attractive approach because of the high quality of sequence information generated, and its time consuming performance compensate the price of more advanced technologies such as pyrogenomics and similar.

Cloning refers to the procedure of isolating a defined DNA sequence inside a competent cell and obtaining multiple it in vitro copies thanks to cell replication. On the first place a fragment of DNA from the PCR product is introduced into the purified DNA genome of a self-replicating genetic element, usually a plasmid, known as cloning vector, **ligation**. Plasmids are small circular molecules of double-stranded DNA derived from large plasmids that occur naturally in bacterial cells, and can only introduce one single type of DNA into their structure. As can be seen in fig. 35, this plasmid is then inserted into a host, usually genetically modified E. Coli bacteria (competent E. Coli), by applying a thermal or electrical shock to the cell. This process is called **cell transformation**. Those transformed bacteria are thenceforth spread into a nutrient enriched media consisting in agar plates where they grow and become visible isolated in colonies (**cultivation**). This nutrient media contains antibiotics to prevent the opportunistic growth of bacteria from the environment, or those E. Coli which have not assimilated the vector (as this, in turn, contains genes of antibiotic resistance)

After 12-18 h, diverse colonies will have grown over the agar plates. Those colonies are individually regrowth in 96 wells plates filled with liquid media (**isolation**). The result of this liquid regrowth is then submitted to automatic DNA extraction and subsequent sequencing.

TOPO® TA Cloning® Kit for Sequencing, with One Shot® TOP10 Chemically Competent *E. coli*, was used to perform all the process. According to its protocol the first step was to perform the ligation reaction by mixing and incubating at room temperature the ingredients listed in table 25.

Table 5. Ligation reaction

	v (µL)
PCR product	3
pCR™4-TOPO® (plasmids)	1
Salt Solution (1.2M NaCl, 0.06M $MgCl_2$)	1
Water	1
Total volume	6

The transformation process took place by introducing 2 µL of the ligation reaction product in a thawed vial of TOP10, Mach1™-T1R chemically competent cells. After a 30s heat shock at 42 ºC and the immediately ulterior immersion on ice, transformation took place. For better spreading of the cells, S.O.C. medium (Super Optimal Catabolite) was added to the cells vials, and aliquots from 50 to 70 µL were spiked and spread in different agar plates (35 $g \cdot L^{-1}$ LB Agar, (Pronadisa)). Once the colonies were visible, after a period of incubation at 37 ºC, each of them was picked and transferred to an individual well of liquid media (40 $g \cdot L^{-1}$ LB Broth, (Pronadisa)) for their isolation. Those 96-wells plates containing the liquid media were also incubated at 37 ºC, and a replicate of every plate was also performed. After 20 h of incubation plates were centrifuged and liquid supernatant was discarded. By means of Robosec 4204SE automat robot (Applied Biosystems), at Unitat de GenómicadelParcCientific, the centrifuged material from the liquid plates was purified and extracted using Millipore Plasmid Miniprep Kit (Millipore). As a result, a new plate with one single targeted sequence per well was obtained, ready for sequencing reaction.

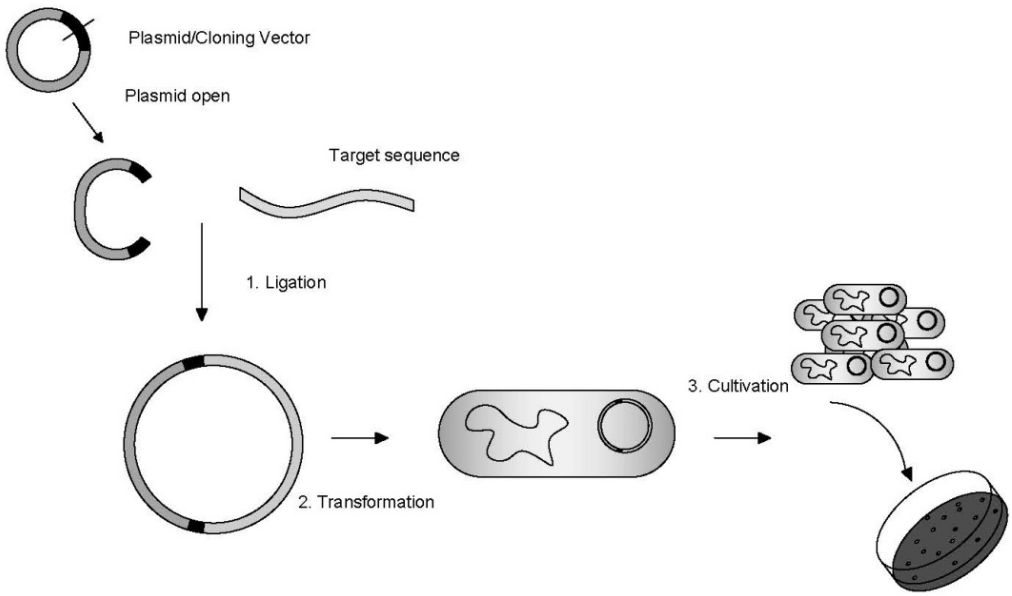

Figure 13. First cloning steps

3.3.7. SEQUENCING

After automatic extraction, one or more plates with 96 wells with individual DNA content from 96 or more different regrowth colonies is ready. The last practical step of this process consists in the sequencing of the content of every one of those wells, "reading and identifying" the DNA molecule present in each well that will correspond to a certain kind of bacteria, unique or present also in other wells (supposedly depending on the abundance in the original sample)[4].

Sequencing consists in a process where the DNA strands from each well, replicated by the growing of the liquid culture, are amplified and read in the presence of dNTPs and a special kind of nucleotide, dideoxyribonucleotide triphosphate (ddNTP), according to Sanger Dideoxy Sequencing. They are identical to typical nucleotides except that, instead of having a hydroxyl group attached to their 3' carbon, they just have a hydrogen atom. These nucleotides are then considered chain-terminating, due to the fact that that hydroxyl group would be needed for continuing the adding reaction. They will also have the peculiarity of being labeled with different colored fluorescent dye or radioactivity (different color or wave length for each four nucleotide). The fact that the addition of a ddNTP interrupts the growth of the chain allows the sequencing process to determine the order of nucleotides in a strand of DNA.

Most DNA sequencing today is *cycle sequencing* which combines the original methods of DNA sequencing with the heating and cooling cycles of PCR. However, apart from the presence of ddNTPs on the reaction mixture, there is an important difference with

[4] If there was a high abundance in the sample, more replicas would be obtained after PCR reaction, so more colonies of E. Coli will grow with that insert in their plasmids, so wells of the liquid culture will have that molecule being replicated, son sequencing process will find the same molecule in many wells.

the conventional PCR described before. This is the use in each run of just one primer, instead of a couple of R and F primers, as can be seen in fig. 36. For obtaining better quality data, the sequencing is performed in two runs with a F and a R primer separately, and then the resulting sequences, which are expected to be complementary, can be aligned and constitute a confirmed a single strand sequence called **consensus** that could be considered as corrected by comparison of the two complementary strands.

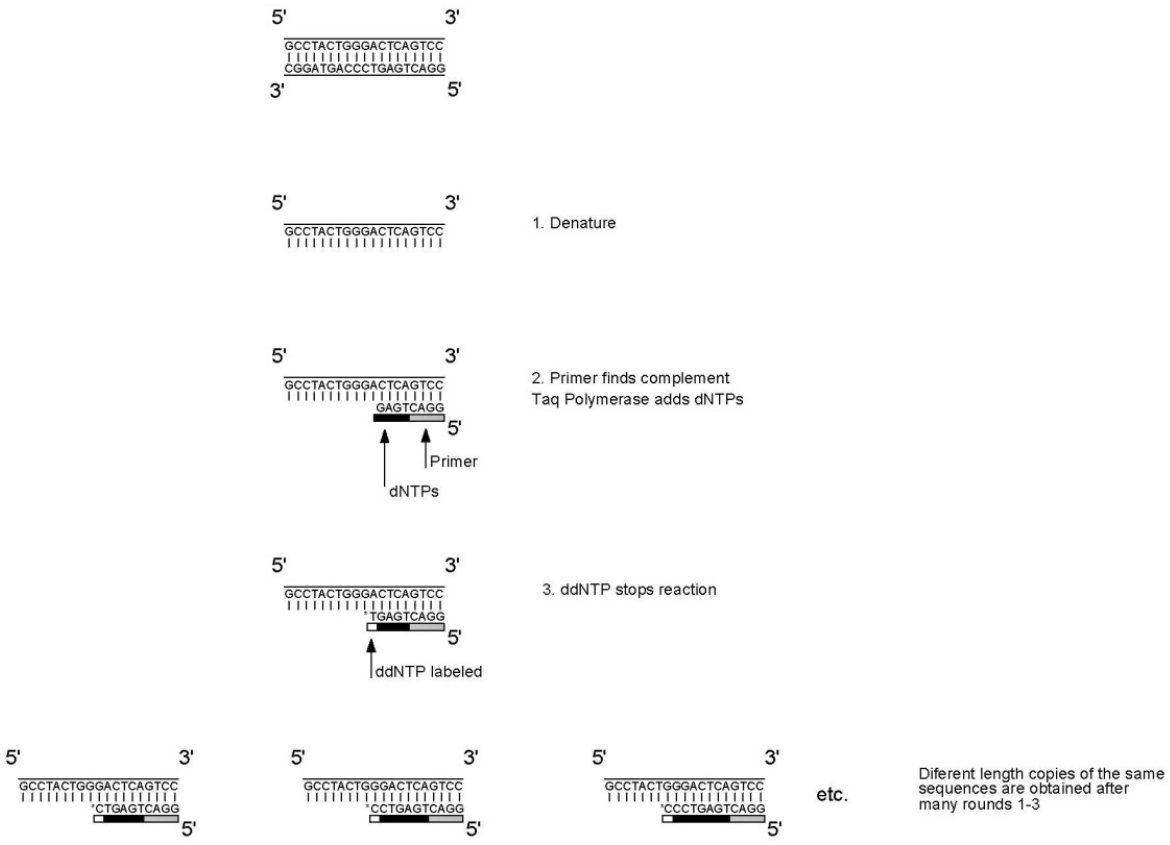

Figure 14.DNA sequencing with R primer.

Among different sequencing methods employed, chain-termination method was used in this thesis, by means of BigDye® Terminator v3.1 Cycle Sequencing Kit. The kit contains the reagents needed for sequencing, pre-aliquoted and ready to use. It utilizes fluorescent labeled ddNTPs, thus the termination is detected by the fluorescence of the terminating nucleotide, and the presence of all the ddNTPs and dNTPs in the same mixture allows performing just one reaction, instead of the four reactions needed for traditional Sanger Sequencing. The recipe used during this thesis was optimized with the collaboration of Unitat de Genómica del ParcCientific de Barcelona. Table 26 recaps the reagents volume.

Table 6. Sequencing PCR Recipe

	v (µL)

Big Dye terminator Ready Reaction Mix	1
Buffer	3.5
Primer (5mM)	1
Water	12.5
DNA Template	2
Total volume	20

For sequencing, a smaller section of the PCR sequence was chosen, the one comprised between primers V3R and V3F (Yang et al. 2009), common to most of bacteria and which includes enough inner hypervariability for genotyping-fingerprinting. Table 27 indicates the sequences that characterize both primers.

Table 7. Sequencing primers

V3R	5'-CGTATTACCGCGGCTGCTG-3'
V3F	5'-CCAGACTCCTACGGGAGGCAG-3'

Thermal reaction was also performed in the ABI 9700 (Applied Biosystems) thermocycler, while the "reading" took place by means of ABI PRISM 310 Genetic Analyzer (Applied Biosystems) at ParcCientífic de Barcelona. This device counts with a system of capillary electrophoresis together with an optical system that allows it to recognize the size of every replicated strand (by the electrophoresis principle) and the terminating nucleotide (by the fluorescence of ddNTPs). Thank to this, the data acquisition system detects, for each well, signals from the different replicated fragments of the same sequence and the software builds up a chromatogram in which each peak identifies a different nucleotide of the sequence. Table 28 summarizes cycling set up for sequencing, while fig. 37 pretends to be a schematic explanation of the information that gets the software and how it converts it into the chromatogram that reveals the whole replicated sequence.

Table 8. Cycling program for sequencing

T (ºC)	t (s)	
from 20 to 96	60	
96	10	35
35	5	cycles
60	240	
4	∞	

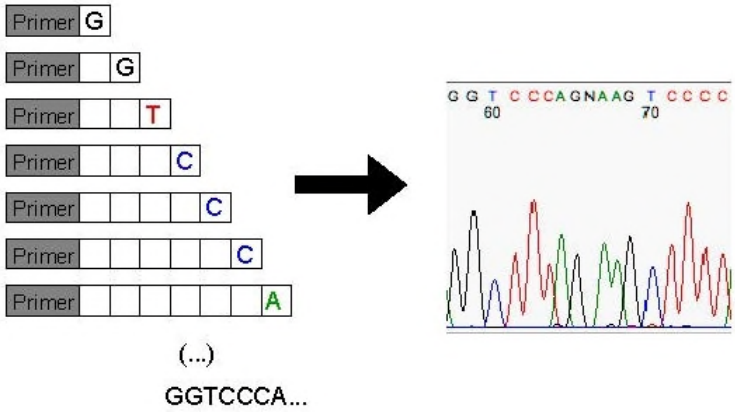

Figure 15.Schematic representation of the data acquisition of a sequencing device and its output information. N is the information the software gives when the data are confusing, two overlapping peaks, the lack of fluorescence, etc.

3.3.8. RESULTS ANALYSIS

Starting from the information in the chromatograms associated to each well, the nucleotide sequence of that particular DNA fragment can be obtained. According to the chromatogram itself and the experience of the analyzer, some mistakes that can be found in the sequence due to the replication process can be corrected. Once the sequence is revised, converted into a .txt file it is processed in different ways.

Fig. 38 gives an idea of which region is sequenced in each round. For phylogenetic information only the fragment of the sequence from the beginning to where the sequence is complementary to the opposite primer to the one with which sequencing has been done in needed. This fragment corresponds to the targeted V3 region. The rest of the sequence would contain V3 external region and plasmid sequences. Once the useless pieces of the sequence are eliminated from V3R and V3F PCRs product sequences, the resultant arrangements are supposed to be quasi-identical. With a software called Lasergen® MegaAlign™ (DNAStar), both sequences are compared and aligned via pairwise, the discrepancies between series can be corrected obtaining a consensus sequence that is employed for further analysis.

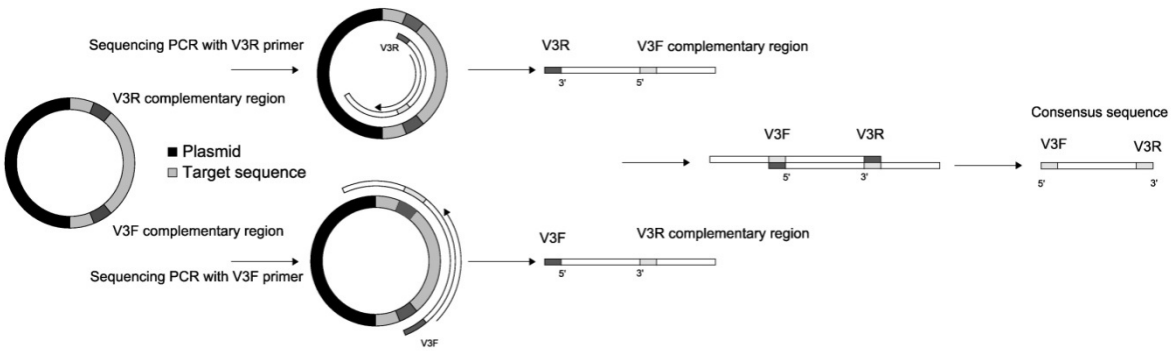

Figure 16. Simplified sequencing PCR process.

Once the consensus sequences are ready, Basic Local Alignment Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi, National Centre for Biotechnology, USA) can be used to find regions of local similarity between the consensus sequences and previously recorded sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches with other bacteria found in previous works by the scientific community. Once a sequence is introduced in the program, it gives a list of bacteria which 16s rDNA contain a sequence similar to the one introduced. As higher similarity value is as closer to that bacterium is the organism from our initial sample from which the introduced sequence came from.

BLAST itself, together with the taxonomic database of National Centre of Biotechnology, www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html, allows inferring functional and evolutionary relationships between sequences as well as help identify members of gene families, enabling the construction of a graph with the distribution of the whole bacterial population that was supposed to be in the initial sample, as it is shown in fig. 39.

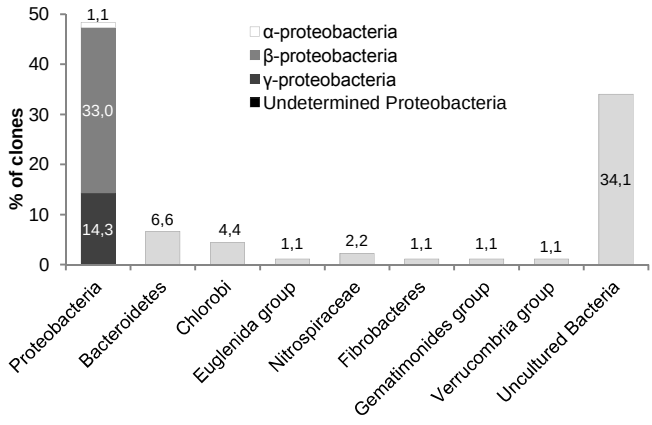

Figure 17. Example of taxonomical distribution

Finally, all the consensus sequences obtained from one samples can be compared among themselves in what it is called a phylogenetic tree, fig. 40, by means, once again, of DNASTAR Lasergen® Megalign™ software. This diagram allows viewing evolutionary relationships predicted from the multiple sequence alignment among the sample. The length of each pair of branches represents the distance between sequence pairs, while the units at the bottom of the tree indicate the number of substitution events.

Figure 18. Phylogenetic tree, from (Todar 2009)