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

Mª del Mar Micó Reche

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Programa de doctorat: Ciència i Tecnologia de Materials

Memòria de tesis doctoral:

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

La Dra. **CARMEN SANS MAZON**, professora titular del Departament d’Enginyeria Química de la Universitat de Barcelona,

CERTIFICA QUE:

El treball d’investigació titulat “**PHOTO-FENTON AND SLOW SAND FILTRATION**” constitueix la memòria que presenta la Enginyera Química **Mª del Mar Micó Reche** per a aspirar al títol de Doctor per la Universitat de Barcelona. Aquesta tesi doctoral ha estat realitzada dins del programa de doctorat “Ciències i Tecnologies de Materials”, en el Departament d’Enginyeria Química de la Universitat de Barcelona.

I perquè així consti als efectes oportuns, signo el present certificat a Barcelona, 12 de juliol de 2013.

Dra. Carme Sans Mazon

"

"Beberemos y desbeberemos la misma agua mil veces"

Dr. Takashi Asano, Department of Civil and Environmental Engineering,
University of Californi, Davis

La Vanguardia, 05/10/2011

A mis padres

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Quisiera dar mi más sincero agradecimiento a mi directora de tesis, la Dra. Carme Sans, por haberme perimitido participar en este proyecto y por haber puesto todo de su parte para que pudiera desarrollar mi vocación investigadora durante estos años, así como por su apoyo y supervisión. Muchísimas gracias también al Dr. Santiago Esplugas por recibirme en su grupo de investigación, y a todos sus miembros por haberme acompañado en el trayecto hasta aquí.

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ABSTRACT

This thesis is framed in the collaboration between the Chemical Engineering Department, from the University of Barcelona, and the R+D Department from the company Acciona Agua S.A.U., under the umbrella of CENIT-MEDIODIA Project. This initiative, founded by CDTI-Spanish Ministry of Science and Innovation comprised a consortium of companies and research centres that focused their innovative efforts in the development of a new concept of Advanced Hydroponics Greenhouses.

These facilities, highly automatized, built with leading-edge materials, aspire to go beyond the limits of traditional protected agriculture with higher control of the production, by-products, plagues, etc.; increasing productive periods; and the efficient use of renewable sources of energy and water.

Precisely the collaboration between the University of Barcelona and Acciona Agua was focused on optimizing greenhouses hydric resources. The functionality of a combined treatment had to be assessed, when applied to the discard stream of a recycling system of the Advanced Greenhouse leachates. The coupling consisted on an Advanced Oxidation Processes (AOP), photo-Fenton, based on hydroxyl radical oxidative potential, produced by the interaction between Fe^{2+} and H_2O_2, and a slow sand filtration column acting as a bioreactor. The recycling system will require the synergy of chemical and biological processes to be able to work efficiently with the particular characteristics of greenhouses effluents: high salinity content and the presence of pesticides.

The two recycling strategies proposed by the project defined two salinity thresholds that the coupled system should be able to cope with. The first strategy proposed a simple semi-closed system that recycled nutrient solution from the hydroponics crops until a maximum value of 11 $mS \cdot cm^{-1}$, phytotoxicity limit. Part of the current was then diverted to be treated by the integrated system. The second strategy introduced reverse osmosis membrane technology that concentrated that diverted stream, sending the permeate for its reuse directly to the greenhouse, while the brine had to be treated by the coupled process. In this case the maximum level of salinity in the effluents could reach conductivities close to those for seawater, around 50 $mS \cdot cm^{-1}$.

According to this, the experimentation was performed dealing with three aspects regarding the coupled system: photo-Fenton performance, bioreactor performance (biocompatibility of the effluents was firstly tested), and molecular biology tools (MBTs) applied to the characterization of the biomass developed in the treatment. This issue was of extreme interest given its novelty in the field of wastewater, and the wide potential of MBTs.

As the different points of view of the integrated process between photo-Fenton reaction and slow sand filtration column were considered, the behavior of three structurally different pesticides was also monitored: methomyl, a carbamate insecticide; imidacloprid, a neonicotinoid insecticide; and fosetyl-Al, an organophosphorous fungicide.

The performance of photo-Fenton reaction was essayed in order to improve the knowledge regarding this treatment technique. On the first place, this AOP and the ozonation process were compared. Results shown that increasing toxicity of ozonation effluents confirmed the choice of photo-Fenton as the most adequate treatment for pesticide polluted effluents. Experimental design criteria allowed then to determine optimal working conditions depending on the content of the reaction media, and enabled to prove the existence of endogenous catalyst inhibition in the presence of fosetyl-Al. Salinity essays were finally performed, yielding positive results even for highest conductivity effluents. Those positive results were also reflected in the increase of the biodegradability of the treated effluents, what leaded to the next step of the research.

Biocompatibility of pretreated effluents was essayed by means of sequencing batch reactors (SBBR). These devices were used to show how photo-Fenton indeed increased biodegradability of the effluents, and how it grown until a certain point when more hydrogen peroxide did not lead to better results. They were also utilized to assess the biocompatibility of high salinity pretreated effluents, as a first step towards the coupling with the slow sand filtration at high conductivities. Results obtained were extremely encouraging, given that even for the highest salinity concentrations (10 and 50 $mS\cdot cm^{-1}$), the performance of the bioreactor achieved an organic content reduction for more than 80% of the loaded concentration, which compared to the 10-20% removal achieved by photo-Fenton, justifies the need of combining both treatments.

Guided by those positive results, the load of the slow sand filtration column with different salinity pretreated effluents was performed. Also positive results were obtained. The achieved elimination of the organic content was more than 75% when loaded with 10 $mS\cdot cm^{-1}$ effluent, and the refractory fraction (the remaining organic matter that cannot be oxidized either by photo-Fenton reaction or by the biomass metabolism) was the lowest also for this high conductivity.

Molecular biology tools used in this thesis were based on cloning and sequencing techniques of 16S rRNA genes. They allowed characterizing the bacterial population of one of the assessed SBBRs and of the different loading stages of the slow sand filtration column. The results showed how crucial they are to understand, at least a part, of the complex microbial environment developed in these engineered systems, and how traditional culture dependent techniques have became obsolete. They showed how with the increase of salinity, the population in the slow sand filtration column lossed diversity, despite the fact that the performance of the column was still proficient. This fact stated how a very different microbial consortium could be developing the same functions as others.

According to obtained results, it could be finally concluded that the coupling between photo-Fenton reaction and slow sand filtration column could be an effective treatment alternative for implementing the recycling strategies of hydroponics greenhouse leachates proposed by CENIT-MEDIODIA Project. For its part, MBTs were revealed as powerfull tools to characterize microbial population and increase the understanding of the bioreaction taking part in bioremediation.

THESIS DIRECTOR'S REPORT AND LIST OF PUBLICATIONS

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Dra. CARME SANS MAZÓN, Professor from the Chemical Engineering Department of the University of Barcelona, and director of the PhD thesis of Mª del Mar Micó, issues the following report related with her participation in the publications derived from this PhD thesis and included in this report:

All the major experimental work and results discussion included in the publications and congresses communications included in this thesis was entirely developed in the Chemical Engineering Department of the University of Barcelona by Ms. Mª del Mar Micó, under the supervision of her PhD director and the researchers of the I&D department of ACCIONA AGUA S.A.U., Mr. Jorge Malfeito and Dr. Jordi Bacardit.

Other institutions and collaborations, if any, are detailed below each article:

I. *Micó MM, Bacardit J, Sans C (2010a) Experimental design applied to photo-Fenton treatment of highly methomyl-concentrated water. Water Sci Technol 62:2066-2074. doi: 10.2166/wst.2010.522.*

II. *Micó MM, Chourdaki S, Bacardit J, Sans C (2010b) Comparison between ozonation and photo-fenton processes for pesticide methomyl removal in advanced greenhouses. Ozone-Sci. Eng.,32:259-264.*

    Ms Micó took under her supervision an Erasmus student, Ms. Stella Chourdaki, who collaborated in some of the experimental part of the work, mainly related with the analytical monitoring of the processes.

III. *Micó MM, Bacardit J, Carme S (2009) Photo-Fenton reaction applied to imidacloprid highly polluted water removal. 1st International Workshop on Application of Redox Technologies in the Environment.*

IV. *Micó MM, Zapata A, Maldonado MI, Bacardit J, Malfeito J, Sans C (2013a) Fosetyl-Al photo-Fenton degradation and its endogenous catalyst inhibition. Submited to J. Hazard Materials*

Part of the results included in this work were obtained during a research stage that Ms. Micó did at the Plataforma Solar de Almería. Although she did the entirely experimental work, she had the collaboration of Ms. Zapata and Dr. Maldonado in the supervision of her work.

V. *Micó MM, Bacardit J, Malfeito J, Sans C (2013b) Enhancement of pesticide photo-Fenton oxidation at high salinities. Appl Catal B-Environ 132–133:162-169. doi: 10.1016/j.apcatb.2012.11.016.*

VI. *Micó MM, Bacardit J, Malfeito J, Sans C (2013c) Biotreatment as part of an integrated system for pesticides removal in high salinity effluents: Performance and biomolecular assessments. Submitted to Chemosphere.*

Dra. Carme Sans Mazón

Barcelona, 8th July 2013

ACRONYMS AND SYMBOLS

Hereby, a list of most used acronyms and symbols.

[-]	concentration of substance "-"
$[-]_0$	initial concentration of substance "-"
$[-]_f$	final concentration of substance "-"
2,4-D	2,4-dichlorophenoxyacetic acid
AOP	advanced oxidation process
BFC	bioaccumulation factor
BLAST	Basic Local Alignment Search Tool
BOD5	biochemical oxygen demand at 5 days
bp	base pairs
COD	chemical oxygen demand
DDT	dichlorodiphenyltrichloroethane
DO	dissolved oxygen
DOC	dissolved organic carbon
EC	electrical conductivity
EU	European Union
H'	Henry's law constant
HPLC	high performance liquid chromatography
K_d	sorption coeficient
K_{oc}	octanol/water partition factor
LC	liquid chromatography
MS	mass spectrophotometry
PCR	Polymerase Chain Reaction
$Q_{50\%}$	energy consumed for degrading 50% of the target compound
RD	Royal Decree
S	solubility
SBR	sequencing batch reactor
SSF	slow sand filtration
$t_{50\%}$	half-life time
TOC	total organic carbon
UV	ultra violet
VSS	total volatile suspended solids
WWTP	wastewater treatment plant

1

INTRODUCTION

1.1. CENIT MEDIODIA PROJECT

The research carried out over this thesis was part of the collaboration between the Chemical Engineering Department, at the University of Barcelona, and the R+D Department from the company Acciona Agua. This collaboration was framed in the CENIT-MEDIODIA Project, founded by CDTI-Ministry of Science and Innovation of the Spanish Ministry and coordinated by Repsol YPF. This project comprises a consortium of 14 companies and 21 research centers, including Unviersity of Barcelona.

The main objective of this Project was to perform an extensive work of research and innovation in the field of protected agriculture, in order to obtain multidisciplinary knowledge for the development of a new concept of Advanced Hydroponics Greenhouses. This concept describes greenhouse facilities highly automatized, built of innovative materials, which will allow the producers to increase their planning capability, and production control, increasing the number of cultivable varieties, extending productive periods, and insuring the efficiency in water and energy consumption. In order to fulfill all those requirements, a series of tasks were distributed among the participants that were grouped in 4 different working areas as depicted in fig. 1.

Precisely, the partnership between Acciona Agua and University of Barcelona was centered in the 3rd activity, Centralized Renewable Supplies, in the subtask focused on the reuse of residual leachates originated in Advanced Greenhouses, in order to ensure maximum efficiency of water resources.

During the first steps of the project, primary objective of the collaboration was established. The functionality of a combined Advanced Oxidation Processes, AOPs, and a bioreactor had to be assessed when applied to the discard stream of a recycling system of the Advanced Greenhouse lixiviates. This recovering system, which will be described later, would require the synergy of chemical and biological processes to be able to work efficiently with the particular characteristics of greenhouses effluents: high salinity content and the presence of pesticides.

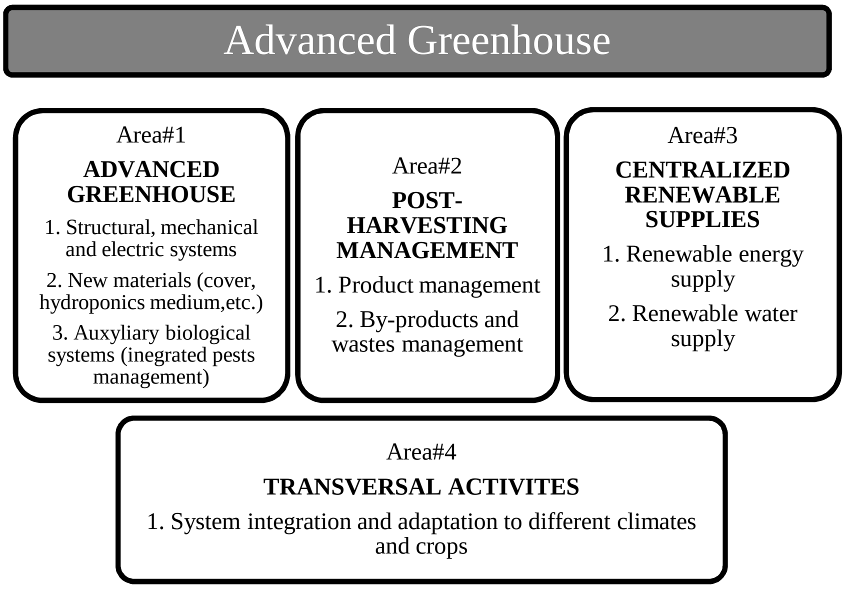

Figure 1. Working Areas and tasks of CENIT-MEDIODIA project.

In addition to the research work required and directly reflected in the results of this project, this thesis includes another two significant features.

- An important work of contextualization of the main issues to be addressed over the investigation. From establishing the state of the art of photo-Fenton reaction applied to the decontamination of pesticides, to find examples of coupled system that encouraged the basis of the collaboration.
- Molecular Biology Tools, MBTs, were applied to characterize bacterial population of the bioreactors assessed in this work. This set of extremely innovative technologies, recently introduced in the researching group, was tuned for the special necessities of the project, strongly influenced by the salinity content of the effluents.

1.2. WATER REUSE

Communities across the world face water supply challenges due to increasing water demand, contamination of groundwater, and dependence on single sources of supply (Wade Miller 2006). The climate warming is underlining the profile of droughts and floods (Oron et al. 2007). Even in northern European countries, traditionally considered as “water-rich”, like United Kingdom, Belgium or the Netherlands, the per capita water availability is approximately in the same range as for dryer regions. Their supremacy position in water resources has been challenged in the last decades by growing water stress, both in terms of water scarcity and quality deterioration. In fact, nowadays, half of the European countries, representing almost 70% of the population, are facing water stress issues (Mediterranean Wastewater Reuse Working Group, EUWI 2007a). Population pressure increase is precisely another fact to blame. Currently, 1.4 billion

(109) people live in water basins where the water usage rates exceed recharge rates (Seckler et al. 1999). By 2030, the number of people in the world living under severe water stress is expected to increase by 1 billion from the 2005 baseline to an estimated 3.9 billion people (47 % of the world population). According to this, water concern is neither a new issue nor a problem just affecting Europe. Kyoto (1997), Hague (2000), Bonn (2001) Conferences and the Johannesburg World Summit on Sustainable Development (2002), already recognized the need for better water management worldwide.

Water has long become a limited resource in an expanding global economy and population, which have accelerated the search for alternative sources (Hartley 2006). In this Global scenario, many water resource professionals believe that **reclaiming water** after its treatment in an adequate wastewater treatment plant is an important and underutilized element of sustainable water resource management (Hartley 2006). Reuse of reclaimed water and recycling[1] address these challenges by resolving water resource issues and creating new sources of high-quality water supplies for urban, industrial, agricultural, and environmental uses (Haering et al. 2009).

Urban reuse

- Ornamental landscape and golf course water features
- Fire protection
- Dust control and concrete mixing on construction sites
- Vehicle and window washing
- Toilet flushing in public, commercial, and industrial buildings

Industrial reuse

- Cooling water
- Boiler make-up water
- Industrial process water
- Intentional indirect potable reuse[2]

Agricultural reuse

- Irrigation

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[1] *Reclaimed water implies the recovery of water from domestic, municipal and industrial WWTP (wastewater treatment plant) that has been treated to meet certain standards that allow safe reuse* (Haering et al. 2009). *Recycled water does not strictly imply the treatment prior to reuse, although it could also be referred to the same concept.*

[2] *Intentional indirect potable reuse means that reclaimed water is discharged to a water body where it is then purposefully used as a raw water supply for another water treatment plant. This occurs unintentionally in most rivers, since downstream water treatment plants use treated water discharged by upstream wastewater treatment plants. Meanwhile, direct potable reuse refers to the use of reclaimed water for drinking directly after treatment.*

Environmental reuse
• Habitats restoration or enhancement • Groundwater recharge • Augmentation of potable supplies

Water reuse enables practitioners to manipulate the water cycle, thereby creating needed alternative water resources and reducing effluent discharge to the environment. It has become an essential component of integrated water resources management and sustainable development, not only in dry and water deficient areas, but in water abundant regions as well. Even in areas with abundant precipitations where water supply may be costly due to extensive transportation and/or pumping, water reuse has also become an important economic alternative to developing new sources of water (US Environmental Protection Agency 2004). Reclaiming water allows the conservation and rational allocation of freshwater resources. MED-EUWI Working Group on Water Scarcity and Droughts confirmed treated wastewater reuse as a way of addressing long term imbalances between demand and water supply. It increases the total available water supply and reduces the need to develop new water resources and therefore provides an adaptation solution to climate change or population density induced water scarcity by increasing water availability. The use of treated wastewater reduces the amount of discharges and therefore the level of nutrients or other pollutants entering waterways and sensitive marine environments. Mediterranean-EUWI Working Group on Groundwater recognized the importance of its potential impact on the quality of groundwater. Water reuse can also provide a mitigation solution to climate change. The reuse of water in the manufacturing industry reduces fresh water demand, recovers heat and reduces industrial wastewater production. In the case of agricultural usage, it could reduce the need for chemical fertilizers. Furthermore, reclaimed water can be used to enhance the environment through the streams, wetland, and ponds, or to recharge aquifers (preventing saltwater intrusions by barrier formation). Regarding to all that, future potential for reclaimed treated effluent is enormous nowadays and it is considered a profitable field of investment, not only in global facilities, but also in local recycling-treating-reusing systems. According to diverse market surveys, (Freedonia Group 2009; Global Water Intelligence 2009; Global Water Intelligence 2010)the growing of the sector of reuse reclaimed water is expected during the following decades. It is expected that between 2009 and 2016 investment will grow by 19.5 % annually, while the installed capacity of wastewater reuse quality will increase from 28 M $m^3 \cdot day^{-1}$ until 70 M $m^3 \cdot day^{-1}$. Furthermore, water reuse provides a wide range of benefits not just for operators but also for communities themselves, which is translated into creating immense value for the public and the environment (Wade Miller 2006). Some social benefits of water reuse include contributing to food security, better nutrition and sustain agricultural usage. It even acts as a cohesion tool that stimulates water agencies and other stakeholders to work closely together using an integrated approach encouraging good practice and benefit the community. It also increases life quality, wellbeing and health through attractive irrigated landscapes in public facilities (Mediterranean Wastewater Reuse Working Group, EUWI 2007b). Furthermore, water reuse avoids environmental controversies prompted by other alternatives against water scarcity, like long distance water transfers, the construction of big dams, desalination, etc.

AQUAREC, was a research project supported by the European Commission under the Fifth Framework Program (1998-2000) for the funding of research a technological development. It contributed to the implementation of the Key Action "Sustainable Management and Quality of Water" (AQUAREC 2010). One of the objectives was identify and monitor all the water reusing programs that were taking place in Europe, more than 200 were categorized in operation as well as may others in an advanced planning phase. That was a large figure considering that in the early 1990’s municipal water reuse was limited just to incidental cases. Much of the development occurred in the coastline and islands of the semi-arid southern regions, and in the highly urbanized areas of the northern regions. AQUAREC surveys showed that the use of reclaimed water is quite different between those two regions: in southern Europe, reclaimed wastewater is reused predominantly for agricultural irrigation (44% of the projects) and for urban or environmental applications (37% of the projects); in northern Europe, the uses are mainly for urban or environmental applications (51% of the projects) or industrial (33% of the projects) (Bixio et al. 2006a).

AQUAREC also included a comparative study of world data enabling the mapping of water reuse projects around the world and the sectorial use of that water. The mapping outcame that quite a few countries practice water reuse, among them most South European countries. But also countries in the more humid north-western climate have arrived to a certain degree of water reuse (Hochstrat et al. 2008). The current status of treated wastewater reuse and water stress in Europe is summarized in fig. 2. The figure compares the extent of treated wastewater reuse practice with the severity of water stress, for a number of countries. It is obvious that some water stressed regions have already achieved a rather mature treated wastewater reuse practice (e.g. Israel and the Canary and Balearic Islands in Spain), whereas others are still in the phase of expanding their activities (e.g. mainland Spain and Italy). This represents a semi-quantitative assessment and partly reflects the (demand driven) improvement of the framework conditions, such as regulation, and the degree of institutional organization relevant for the establishment of reuse (guidelines, financial support etc.). Strictly data correspond to 2006, it is expected that countries like Germany and France have improved their reuse range nowadays.

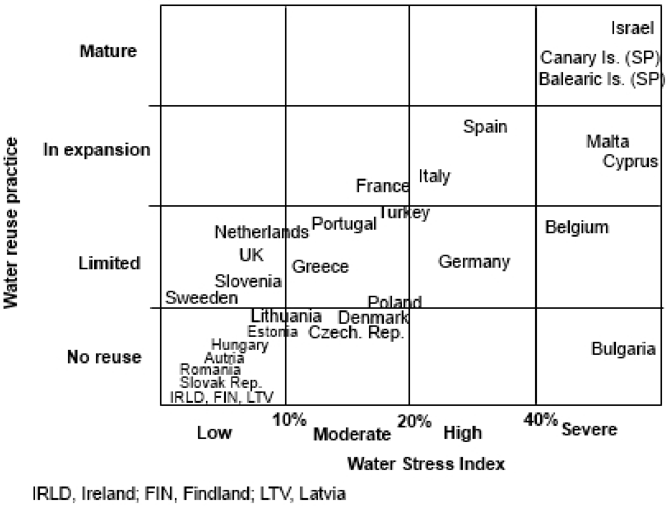


Figure 2. Representation of water reuse practice in European countries and Israel versus their water stress index. Based on (Bixio et al. 2006b)

Fig. 3 shows the reuse patterns in different world regions for the use of reclaimed water. It shows agricultural irrigation mainly focused reuse of reclaimed water in Europe and California, contrasted to the more diverse pattern in Japan. Meanwhile Australia has developed a quite balanced pattern (Hochstrat et al. 2008). Israel is a worldwide reference in the reuse of wastewater. From decades, this country reuses wastewater in agriculture, in a way that allows achieving those mentioned important environmental and financial benefits.

Intrinsic environmental risks on water reuse have also to be taken into account. Hazardous or toxic waste and salts from industry and salt leaching processes in agriculture can endanger the quality of the reclaimed water and create negative effects on the environment. For example, emerging pollutant such as pharmaceuticals and endocrine disruptors may affect the ecological or human health. Undertreated effluents could spread of human or animal pathogens and eutrophication nutrients. Furthermore, the impact of the concentrated wastes that could derive from the reclamation treatments need also to be carefully managed.

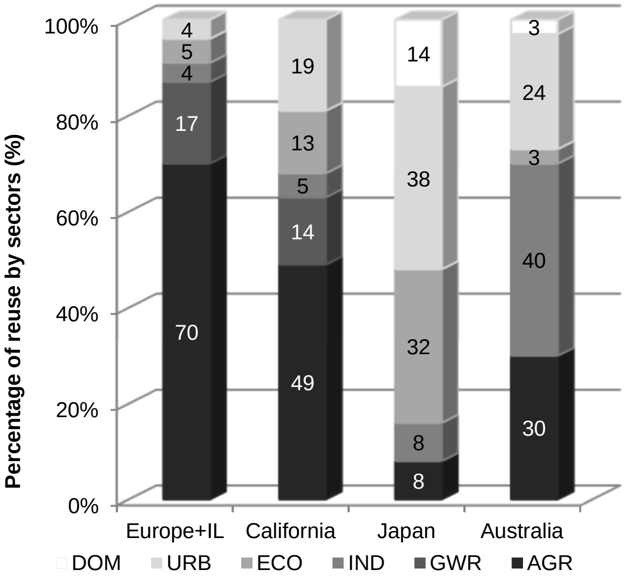


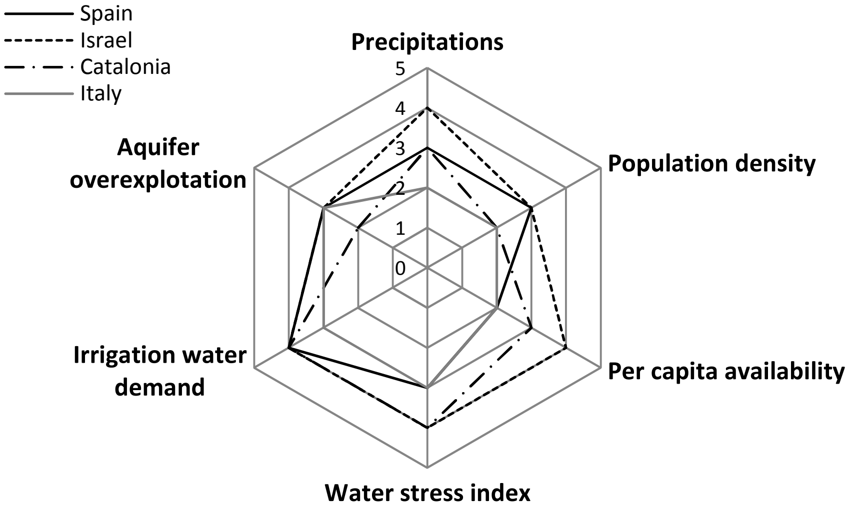
Figure 3. Comparison of water reuse patterns in different regions according to AQUAREC conclusions (IL: Israel), where AGR: agricultural irrigation, GWR: groundwater recharge, IND: industrial uses, ECO: ecological/environmental applications, URB: Urban applications, DOM: domestic uses. Based on (Hochstrat et al. 2008), sources (AATSE 2004; Asano 2000; Hochstrat et al. 2006).

1.2.1. WATER REUSE IN SPAIN

Fig. 4 illustrates the situation for some Mediterranean countries (Spain, Italy and Israel) and regions with regard to water management relevant parameters such as precipitation, population density, water use and alike. These regions score high for most categories, indicating a multi-factor water stress (Mediterranean Wastewater Reuse Working Group, EUWI 2007a).

Total volume of reused treated wastewater in Europe is 964 Mm^3/a, which accounts for 2.4% of the treated effluent. Spain accounts for its largest proportion (347 Mm^3 /yr) (Mediterranean Wastewater Reuse Working Group, EUWI 2007a). Although both planned and incidental water reuse have been taking place in Spain for decades, particularly in coastal Mediterranean areas and in the Balearic and Canary islands, planned water reuse became a viable option as a consequence of the 1st International Symposium on Water Reclamation and Reuse held in Costa Brava in 1991. Since then, numerous projects have been implemented across the country, mainly serving agricultural irrigation as well as landscape irrigation, environmental restoration, and urban uses such as street cleaning, urban landscape irrigation, boat washing, and fire control (US Environmental Protection Agency 2004).

Royal Decree 1620/2007 is nowadays the principal water reuse regulation for Spain. It established the legal regime for the reuse of treated water, including the basic conditions for reuse of treated water, quality standards and the proceedings for initiatives or plans from public authorities' initiatives. It represented an important advance to standardize water reuse practices despite the large cost of implementation. It contributed to the consolidation of water reuse inside the global water resources management (Iglesias et al. 2010).



Precipitations	Population density	Per capita availability
1: >1000 mm 2: 701-1000 mm 3: 501-700 mm 4: <500 mm	1: < 50 inh/km^2 2: 50-200 inh/km^2 3: 201-500 inh/km^2 4: >500 inh/km^2	1: >5000 m^3/inhyr 2: 2501-5000 m^3/inhyr 3: 1500-2500 m^3/inhyr 4: <1000 m^3/inhyr
Water stress index	**Irrigation water demand**	**Aquifer overexplotaition**
1: <10% 2: 10-20% 3: 21-40% 4: >40%	1: <10% 2: 10-25% 3: 26-50% 4: >50%	1: not observed 2: slight problems 3:obvious problems 4: big problems

Figure 4. Spider chart comparing some major water management characteristics of Mediterranean countries and regions (Mediterranean Wastewater Reuse Working Group, EUWI 2007b). *"inh" stands for inhabitants.*

Central and autonomic governments have been doing noticeably efforts to spread the reuse of reclaimed water for the last two decades. These initiatives were finally recognized, reunited and organized in 2009 under a nationwide project entitled: *National Plan of Reclaimed Water Reuse*. The objectives of the plan were both strategic and environmental. The main strategic aims were: achieve "zero discharge" objective in coastal areas; replace inland areas pre-potable water concessions by reclaimed water for uses where feasible; promote good practices of reuse of reclaimed water, estimate future reusability, etc. As environmental objectives, the plan envisaged the change from the traditional approach of "supply", founded on the basis of large hydraulic infrastructures, to new strategies of water resources "on-demand management" and the protection of inland and coastal estuaries ecosystems (Ministerio de Medio Ambiente, Medio Rural y Marino 2009). The plan covered the entire Spanish territory and its application reached horizon until 2015. However, after the change of government in late 2011, the plan seems to have stopped, so that the last reference to it that appears in the Official Gazette of the State belongs to November 2011 (accessed 11/11/2012) (Ministerio de Medio Ambiente, Medio Rural y Marino 2011).

Traditionally, major impetus for water reclamation and reuse has been based on the viable alternatives for cost recovery. The highly competitive water markets of the Canary Islands, the highly productive hydroponic crops of the southern Mediterranean coast, and the more recent demands for golf course irrigation, have largely contributed to the expansion of water reclamation and reuse in Spain. Mediterranean coastal cities, like Barcelona and Valencia, with traditional high levels of incidental reuse in agriculture, are seriously considering rehabilitation and expansion of their treatment facilities, to impulse even more water reclamation, and satisfy the water quality requirements associated with environmental and public health protection, and include adopting microbiological quality levels that are nearly comparable to those of drinking water quality.

1.2.2. WATER USE AND REUSE IN AGRICULTURE

The use of water for agriculture has changed the production of crops dramatically in the 20th century. Irrigation helps stabilizing and increasing yields, it allows for higher cropping intensity and more flexibility in cropping patterns. The value of production on irrigated systems is considered to be 2 to 4 higher than under rain fed conditions (de Fraiture 2012), and according to FAO, investing in irrigation development provides insurance against erratic rainfall and stabilizes agricultural output, allows diversification; what translates into increased and less volatile farm incomes (Food and Agriculture Organization of the United Nations 2005). Furthermore, there is a clear link between varied diet and access to water, stating the importance of water for agriculture and derived products (de Fraiture 2012). In the case of UE members government subsidies and Common Agricultural Policy (CAP) fostered the use of irrigation. All these facts made that nowadays irrigation is a major user of water resources, 70% of all freshwater withdrawals (Food and Agriculture Organization of the United Nations 2005), requiring one hundred times more than we use for personal needs (Lenntech B.V 2011).

In the case Iberian Peninsula many agricultural areas are characterized by the aridity of the climate and the persistent shortage and low quality of the available water resources (Fernández and Torrecillas 2012). Though in the last 30 years, 17% of the European territory has suffered drought periods (European Commission 2007); Spain is still the most arid country in the European Union, table 1 summarizes the capacities and real reserves of the peninsula. This situation is not only due to Spanish average precipitation, which amounts to 85% of that in the EU, but because of its great atmospheric demand (natural evapotranspiration due to climate and radiation conditions) (Collins et al. 2009). The scarce water resources are increasingly disputed among different productive sectors because of the expansion of urban, touristic and industrial activities. That

Mediterranean agrosystems are bound to face water scarcity has long been known. But even more severe water restrictions must be expected to increase (Collins et al. 2009).

Table 1. Hydrologic tendency report. Capacity (hm^3) and reserves (%) in peninsular reservoirs (Ministerio de Agricultura, Alimentación y Medioambiente. 2012).

Ambits	**Total capacity of the reservoirs (hm^3)**	**Reserves (hm^3)**	**Reserves vs. total capacity (%)**				
			2011	**2010**	**2009**	**5years average**	**10 years average**
Atlantic Basin	41,693	26,733	64.1	76.8	59.2	57.3	58.7
Mediterranean B.	13,897	7,803	56.1	67.8	57.3	49.9	49.6
Total Peninsula	55,590	34,536	62.1	74.5	58.7	55.5	56.5

However, irrigated area in Spain totaled over 3.7 million ha, about 7% of the geographical area and almost a fifth of the agricultural area. Despite this small relative size, intensity of productive irrigated areas and the importance of invested in them, explains that over 35% of total agricultural GDP is obtained from these lands. Irrigation gets 50% of the final agricultural production in only 20% of the agricultural land, with a gross value of annual output per hectare located between 2500 and 3000 €, equivalent to more than 6 times that of rain fed (Ministerio de Medio Ambiente, Medio Rural y Marino 2008).

While most studies conclude that there are sufficient land and water resources available to satisfy global food demands during the next 50 years, these studies also warn that conventional irrigation systems are already jeopardized by water scarcity, even in regions of the world that traditionally have been considered as humid, that end up sometimes with bloody conflicts related to the competition for surface water rights, the over-drafting of underground aquifers (de Fraiture 2012).

Besides, the expansion and intensification of agriculture has also altered the natural hydrology of surface water. The irrigated area has been increasing, mainly during 1970's, while the total agricultural land area has decreased. In these days, it is estimated that 40 percent of all crops grown in the world today are grown using irrigation (Advameg 2012), consuming annually between 6800-7500 km^3 of water (Chapagain 2006; Rockström et al. 1999). By 2030 FAO predicts that 45% of all food will come from irrigated areas (Molden et al. 2007). This extremely high demands cause the diminishment of the traditionally available water resources by upstream extractions, reducing the accessibility for environmental services, floodplains, and other uses downstream, including irrigation itself. In the case of Spain the most prominent impacts of temperature increase due to climate change forecast a diminished runoff and increased demand of irrigation systems. Even more important is the sensitivity of water resources towards temperature increase and reduced precipitation. For the semi-arid regions in Spain, a reduction of up to 50% of the actual available resources seems possible (de Fraiture 2012).

According to FAO, water scarcity for agriculture is endangering feed production for our overpopulated planet. Unfortunately, this overpopulation is also consubstantial to the enrichment of the wealthy sectors, who demand more and better quality of agricultural commodities that require higher quantities of water during production (e.g. meat, fish, dairy and sugar, require more water than traditional staple foods such as grains and tuber crops) (Molden et al. 2007).

This increasing water scarcity, together with growing wastewater volumes generated, containing excess nutrients, pesticides and other pollutants (Lenntech B.V 2011), and the tendency to increase irrigation demand is making reclaimed water more widely used as a low-cost alternative to conventional irrigation water (Mediterranean Wastewater Reuse Working Group, EUWI 2007b). For that reason those regions where competition for scarce water resources is more intensive, there is growing interest in using recycled, reclaimed, desalinated or reused water, mainly from processed drainage water or sewage water, for different application not just agriculture. These considered non-conventional sources of water still remain marginal in most regions, although they are important for agriculture in some localities within countries, especially near large population centers (recycled sewage wastewater) and coastal areas (desalination), such as being to emerge in some OECD Mediterranean countries (Organization for Economic Co-operation and Development 2010). However, in Spain only 1% of total water for irrigation come from alternative sources like desalinated water or reused (Dirección General del Agua 2010).

The first practices of reusing water for irrigation date back hundreds of years and arrives to our days, it consists on the direct use of raw wastewater irrigation of the crops, which simultaneously solves water shortage and disposal concerns (Oron et al. 1999), especially for low income countries. However in richer countries where wastewater treatment is common, the use of treated wastewater is the rule. It is planned according to water quality objectives that are determined by public institutions, and takes into account environmental and health risks (Pedrero et al. 2010) entailed to the non-treated sewage.

In fact, in Spain most of reclaimed wastewater is devoted to agricultural irrigation (Iglesias et al. 2010). The legislated treatment previous to its use, according to current wastewater reuse regulation, consists of a chemical coagulation and precipitation, granular filtration, and disinfection with ultraviolet (UV), chlorine or a combination of both. Their design takes into account parameters such as salinity, pathogens, turbidity, TSS, organic matter, N- and P-related species, etc.

Regarding to the particular interest of this thesis, literature related to water reuse in hydroponics frame is commonly focused on the reutilization of different grades treated wastewater effluents from municipal WWTP (wastewater treatment plant) (Keller et al. 2005; Nair 2008; Oyama 2008; Rana et al. 2011). Nevertheless, this particular case proposes the possibility of reusing in situ exhausted hydroponics feed solution (see next section for further information about hydroponics). The explored idea consisted on a decentralized system of reclaiming water adapted to the facilities of greenhouses exploitation. Instead of relying on the capability of big community wastewater facilities to treat the fed effluents, the decentralized system is expected to treat the outlet of the agricultural facility and recycle to the system the adapted effluent. This strategy pretends to reduce fresh water consumption, and to throw to the public sewage system the lowest possible volume, fulfilling required standards. In this case, the most important parameters that will be taken into account for the design of the treatment will be the presence of toxic substances as pesticides and metabolites; the organic matter content; salinity and N- and P-based nutrients.

1.3. HYDROPONICS

In hydroponics, traditionally terrestrial plants are grown with their roots supported within an inert (organic or inorganic) medium, such as perlite, gravel, mineral wool, expanded clay or coconut husk, through which an engineered nutrient solution flows continuously. In the case of pure hydroponics the roots can directly hang inside the current itself. However the use of solid medium is pretty common as it provides for a favorable buffer of solution around the root system (Benton Jones Jr. 2004).

Historically these techniques have been practiced for centuries (1627, *Sylva Sylvarum*, by Francis Bacon) inside more or less sophisticated greenhouse facilities, however in the 1800s the basic concepts for the hydroponic growing of plants were established, and in 1930s William Frederick Gericke, from Berkeley University, popularized these methods showing the world his own soilless grown vegetables. One of the early victories of hydroponics occurred on Wake Island, a rocky atoll in the Pacific Ocean used as a refueling stop for Pan American World Airways. Hydroponics was used there in the 1930s to grow vegetables for the passengers. The widespread deployment of U.S. troops in the WW II also promoted the establishment of large hydroponic installations in some other Pacific Islands to supply food to the soldiers. However, the major milestone in the development of economic and commercial hydroponics was the *NFT* concept, which stands for Nutrient Film Technique, developed 1965 by Allen Cooper. Depicted in fig. 5, it consists in a cultivation technique in which plants grow within a channel (impermeable walls) through which the nutrient solution circulates (Domingues et al. 2012). This technique lead to the fact that since 70-80s, soilless growing systems have become increasingly popular among big and small growers given the improve production quality, and provide disease free start to cultivation (Van Os et al. 2002). It is even considered by FAO, as a an option in the struggle against poverty (Marulanda and Izquierdo 1993). In 2003, Alexander (Alexander 2003) stated that hydroponics were growing rapidly everywhere and was expected to be established as a major part our agricultural and horticultural production industries.

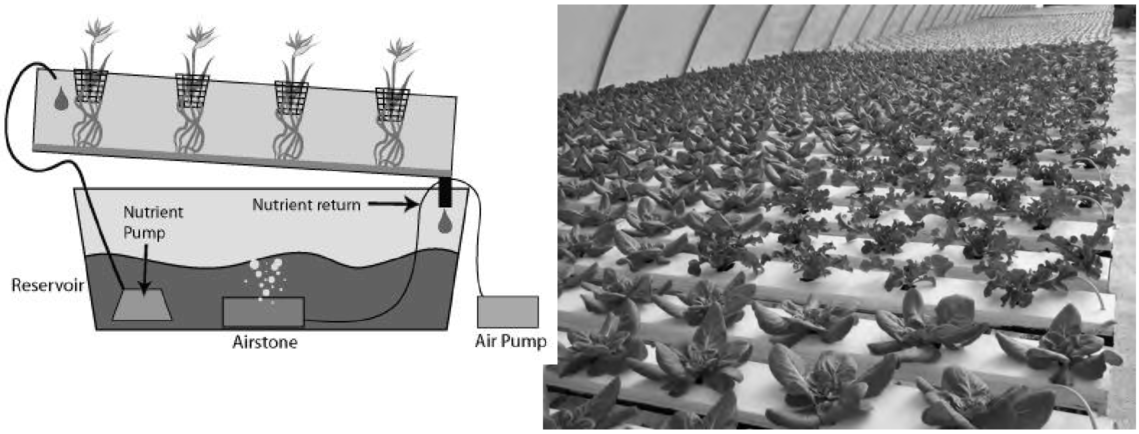


Figure 5. A scheme and an on-field implementation of NFT with lettuce cultivars. (Blogecocityhydorponics.com 01/01/2011) and (Cropking Inc. 2011) respectively.

One of the basic principles for vegetable production, both in soil and in hydroponics system, is to provide all the nutrients the plant needs. Several chemical elements are essential for growth and production of plants, in a total of sixteen elements: carbon, hydrogen, oxygen, nitrogen, phosphorous, potassium, sulfur, calcium, magnesium, manganese, iron, zinc, boron, copper, molybdenum and chlorine. Among the elements mentioned above, there is a division according to their origin: organic C, H, O and minerals; broken down into macronutrients, N, P, K, Ca, Mg, S and micronutrients, Mn, Fe, B, Zn, Cu, Mo, Ni, Cl (Domingues et al. 2012). Commonly used chemicals for the macronutrients include potassium nitrate, calcium nitrate, potassium phosphate, and magnesium sulfate. The concentration of elements can be varied in response to known physiologic stages of the developing plant (Benton Jones Jr. 2004).

In hydroponics crops, absorption of named nutrient is usually proportional to their concentration in the solution near the roots. Furthermore, plants will change the composition of the nutrient solutions upon contact by depleting specific nutrients more rapidly than others, removing water from the solution, and altering the pH by excretion of either acidity or alkalinity. Care is required not to allow salt concentrations to become too high, nutrients to become too depleted, or pH to wander far from the desired value, besides disequilibrium in temperature and pH values could reduce the uptake of certain crucial ions.

The electrical conductivity (EC) is an important parameter for hydroponics crops nutrition. It is proportional to the total content of ions, thus a decrease in EC is accompanied by a proportional fall in the total amount of ions available for absorption by the roots, (although it tends to increase from the bottom to the top of the nutrient solution layer (Rouphael et al. 2006)) affecting unfailingly the development of the plants. It changes nutritional status of the plants (Lauchli and Epstein 1990) as well as many physiological processes (Munns and Termaat 1986). However, moderate salinity level may be beneficial to crops by improving fruit quality and by reducing excessive vegetative vigor of the vegetable crops usually observed during early growth in hydroponics. This allows to take advantage of the quality effect of saline water (high content of dry matter (pulp against juice), reduced sugar, starch, total carbohydrates, Vitamin C and lower nitrate content) (Rouphael et al. 2006). These facts are especially interesting in the case of saline native irrigation waters, such in coastal area of Mediterranean climate (Flowers 1998).

The major advantages of hydroponics systems are the absence of weeds and other soilborne pests, together with no toxic pesticide residue in soil and subjacent aquifers, better use of water, better oxygen and nutrients control, and increased crop quality and yields (Food and Agriculture Organization of the United Nations 2012b). Crops can be grown where no suitable soil exists or where the soil is contaminated with disease. Soilless culture can grow crops safely and in places that were formerly considered too barren to cultivate, such as deserts, the Artic, and even in space (Molders et al. 2012).. In addition, labor for tilling, cultivating, fumigating, watering, and other traditional practices is largely eliminated. Maximum yields are possible, making the system economically feasible in high-density and expensive land areas. Conservation of water and nutrients is a feature of all systems. This can lead to a reduction in pollution of land and streams because valuable chemicals need not be lost, so there will be an especial operating effort on minimizing any losses of nutrients due to leaching (Benton Jones Jr. 2004). Soilborne plant diseases are more readily eradicated in closed systems, which can be totally flooded with an eradicant. More complete control of the environment is generally a feature of the system (i.e., root environment, timely nutrient feeding or irrigation) and in greenhouse-type operations, the light, temperature, humidity, and composition of the air can be manipulated. This allows the possibility of anticipation of harvesting due to the shortening of the plant cycle; showing fast economic return and dispensing crop rotation (Domingues et al. 2012). From the point of view of the plants, the advantages of hydroponics are related to the supply of nutrients, which thanks to the solution are readily available to the plant. Due to their availability, lower concentrations can be used.

However there are also some drawbacks mainly related to economics and labor needed. The original construction cost per acre is great. Trained personnel must direct the growing operation. Knowledge of how plants grow and of the principles of nutrition is important. Introduced soilborne diseases and nematodes may be spread quickly to all beds on the same nutrients tank of a closed system. Most available plant varieties adapted to controlled growing conditions will require research and development. The reaction of the plant to good or poor nutrition is unbelievably fast, so there is a need for continuous monitoring of the operation of the system (Benton Jones Jr. 2004; Domingues et al. 2012).

As was mentioned, conservation of water and nutrients are a feature of all systems. Precisely in reference to water management, depending on the final destiny of the nutrient solution once it has passed through the growing media the hydroponics systems are classified in three different distributions: open, closed and semi-closed system. After passing through the root mass, the nutrient solution is discarded, in what is called an open cycle; or as a closed system in which the nutrient solution, after passing through the root mass or medium, is recovered for reuse. The logical accumulation of salts together with other substances in the closed and semi-closed systems fosters semi-closed systems, in which some nutrient solution is supplied along the circle, while another fraction is discarded and the rest is recycled, thus resulting in water and nutrient losses, and environmental hazard if the discharge is not adequately treated (Massa et al. 2011). According to (Sonneveld and Voogt 2009), "semi-closed" systems leaching fraction may range from 20-30% as it occurs commonly in well-managed open systems. Regarding to this discharges from hydroponics are under water quality regulations, for the possible pesticide content (European Parliament and Council 2000; European Parliament and Council 2006) and especially for the eutrophication affect that nutrients such nitrogen or phosphate can cause in the environment. In Spain, the Nitrate Directive (Jakobsson et al. 2002), which has an special mention to hydroponics, was transposed into national law in 1996, but it was left to the regional authorities to implement Directive until nowadays.

In the case of Spain, the development of hydroponics was strongly related to the highly impact that greenhouse agriculture had in Almeria, a southern province. The abundance of greenhouses in this province is known around the world. Together with the regions of Agadir in Morocco and Antalya in Turkey, this southern Spanish province is one of the most densely covered with greenhouse in the world. Protective agriculture was introduced in Almeria in the 1960s as the application of technological innovations to overcome the limiting factors in this region, poor soil and water scarcity, making the most of the abundant factor, the high insolation (Izcara 2000). This protective agriculture technique allowed that, despite the crisis context lived in Spain, 2011/2012 the province beat its record production increasing it a 8%, with 37,525 ha devoted to greenhouse cropping, 5% more than precedent campaign, and total incomes around 1,400 million of euros, an 8% higher than 2010/2011(Secretaría General de Agricultura y Alimentación 2013). Due to the benefits of the protected and controlled environment, in the 1990s hydroponics crops were also introduced. Although this kind of cropping seemed very minority at the beginning, intensive exploiters are paying more attention to this technology in the last decade. The main reason is the direct control that the producers can exert over the different aspects of the crops growing, temperature, nutrients, aeration, insolation, etc. This helps production to meet every day higher exigencies from the competitive market of fruit, vegetables and ornamental flowers.

1.3.1. STRATEGIES FOR HYDROPONICS WATER REUSE IN GREENHOUSES.

As mentioned the proposal for hydroponics water reuse, which frame this thesis, comprises an on-site decentralized strategy of water reuse as recommended by (Fane et al. 2002; Nelson 2005; Nelson 2005; Oron et al. 1999; US Environmental Protection Agency 2004; Wang 2007). Instead of releasing discarded effluents to the public sewage system and applying treatments for the reuse of this stream in municipal/communal facilities, it is proposed to process the exhausted irrigation effluent (several times recycled) from a hydroponic greenhouse in a self-sufficient system. In this case the water to reuse would have a low content in COD but high content in biorecalcitrant substances (pesticides) and nutrients, in opposition to the most common policy of reusing urban wastewater effluents. The main advantage to the environment and to the surrounding community of reusing these emissions on-site is to prevent non-biodegradable substances and nutrients such as nitrates and phosphates from reaching conventional WWTP as much as possible, which usually are not able to handle with them properly. Another important advantage is the diminishment of fresh water use, thanks to the semi-closed system that reuse proposes.

The operational scheme could be envisaged according to two approaches that start with the introduction of a fresh nutrient solution to the hydroponics system. This current is recovered in a reservoir and recycled to the greenhouse by itself. A certain amount of fresh nutrient solution could be added to the recycling stream, just in the case that it runs off of a critical nutrient. The system is kept working until the conductivity measured in the reservoir reaches the threshold value of 10-12 $mS \cdot cm^{-1}$. According to the works by (Fornes et al. 2007) that conductivity still guaranties the viability of adapted plants development. Then that conductivity is reached in the recycling tank, a new nutrient solution or raw water is introduced in the system, while part of the content of the reservoir is detoured to treatment.

That subsequent treatment should be focused in the readaptation of that detoured current, noticeably concentrated in salts, pesticides and their metabolites. The integration of two treatments is proposed in order to obtain a final current with the requested warranties for sending it to the public sewage system, or to be resent to the hydroponics system. The proposed coupled system comprised a chemical treatment through Photo-Fenton reaction, followed by a biological refining treatment in the shape of a slow sand filtration (SSF), as depicted in fig. 6. Phosphate precipitation stage was conceived as a step to eliminate as much solids and precipitates as possible, especially phosphate salts that are known to hinder photo-Fenton performance enormously.

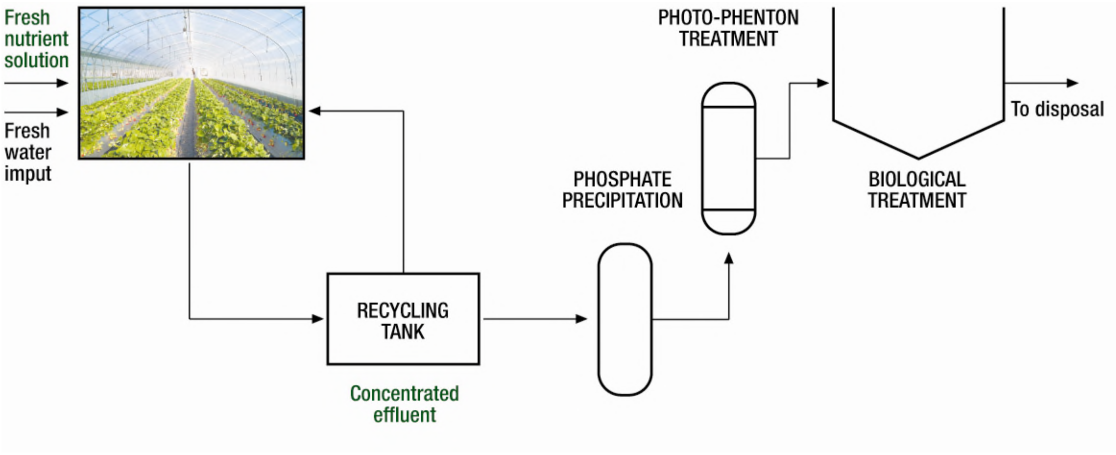


Figure 6: Basic reuse strategy

The second strategy proposes a more sophisticated treatment train that includes a reverse osmosis system. Its permeate stream, free from salts and organic matter, can be used as a water source for preparing new fresh nutrient solution. Meanwhile, the concentrate effluent, with higher conductivity (depending on the recovery of the RO) is sent to the coupled system described before for its readaptation. A scheme of this strategy is represented in fig. 7. In this case, a lower volume of water need to be treated by the coupled system, and as the RO enables the recovery of part of the diverted water, the requirements of the final effluent (after biological treatment) are more focused towards meeting the standards for its emission to the public sewage system than to be resent to the greenhouse cycle.

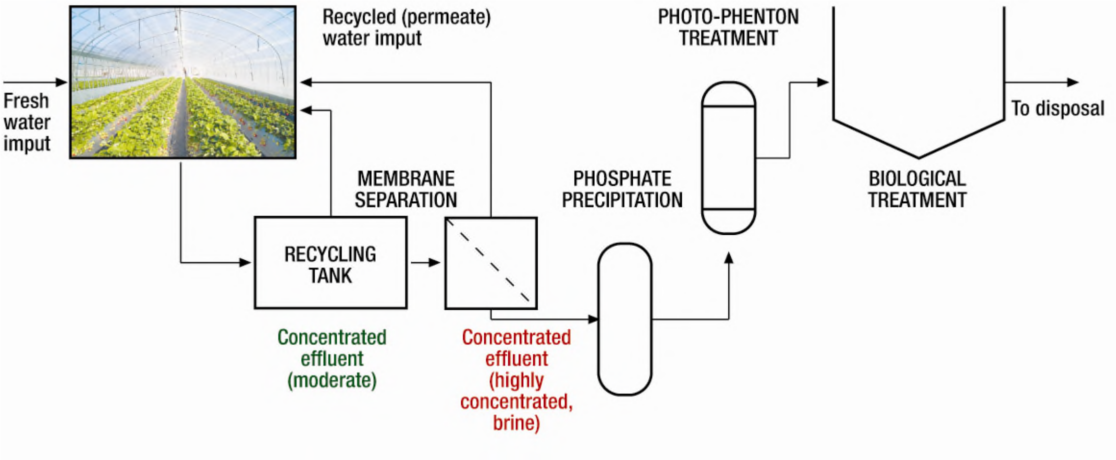


Figure 7: Reuse strategy including a membrane separation stage.

The focus of this thesis is to prove the suitability of the coupled system in this two proposed strategies.

1.4. PESTICIDES

The name pesticides encompasses a number of chemical substances designed to kill, repel, attract, regulate or interrupt the growing of pests in its broadest sense, from agricultural applications to vector disease control. In fact, today, farmers regard pesticides as an essential tool to ensure that they can maintain production of crops of quality and quantity to satisfy an increasing human population. Looking back sixty or seventy years ago, farmers had to rely very much on crop rotations and mechanical weed control with hoes. They also expected that insect pests and diseases could be avoided by choosing a good crop variety that had some resistance to pest damage (Matthews 2006).

Inorganic chemicals, containing copper sulfate, lime sulfur, lead arsenate, mercury salts, etc. were in use since the times of Sumerians (2500BC) and ancient China (1200BC) for fighting insects, mites and body lice. They were used indiscriminately for centuries on field but also to control home pests, in fact Bordeaux mixture, based on copper sulfate and lime is still used against various fungal diseases (Unsworth 2010). It was not till mid-20th Century that their high level of toxicity for humans became evident.

Along the history also some organic chemicals were available, notably the botanical insecticides such as the pyrethrins (extracted from dry *Chrysanthemum cinerariaefolium*), nicotine and rotenone, but they were not widely used due to they were highly susceptible of quick sunlight degradation. On the first decades of 20th century, in Western Europe and North America, the availability of herbicides was a major burst through a time when shortages of labor due to the war were jeopardized farms management. However, the use of synthetic pesticides did not become popular until the end of World War II. During interwar period scientists had been looking for cheap chemicals with persistence in sunlight and low toxicity to man that would kill insect pests quickly, until Dr. Paul Muller in 1938 showed that DDT would indeed fit these specifications. Its use was rapidly extended to agricultural purposes and disease control, and even it was able to suppress diseases breakthroughs in Europe and Asia in those years (Dr. Muller won the noble Prize of Medicine in 1949). Soon in those times, DDT was followed by BHC, aldrin, dieldrin, endrin, chlordane, parathion, captan and 2,4-D, broad-spectrum activity, apparently low toxic for mammals.

However, in 1946 DDT resistance developed by house flies was reported, and a number of reports of harming non-target plants and animals, and residues issues were reported. Throughout most of the 50’s, consumers and most policy makers were not overly concerned about the potential health risks in using pesticides. Food was cheaper because of the new chemical formulations, and with new pesticides coming out every year, there were no documented cases of people dying or being seriously hurt by their “normal” use, the Green Revolution of the 1950’s had just started. The new pesticides seemed rather safe, especially compared to the forms of arsenic that had killed people in 1920’s and 1930’s. Global recognition of problems associated to the extended use of this new plaguicides were at the end of the decade, and were brought to public opinion by Rachel Carson, who laid the foundations for the future ecologist movement (Carson 1962).

Despite the arguments against pesticides, their use became more common in every region of the world. Furthermore, in the developed countries, their usage was closely related to the changes introduced in the production and growing method that, in the 50's, doubled the productivity of agriculture in comparison to other sectors of economy. It should be taken into account that even under the present control practices, the crop losses due to insect pests, diseases caused by various pathogens and competition from weeds, were estimated to range from 26 to 40% for food and fibre crops (Peshin 2002), with weeds causing the highest potential loss (Oerke and Dehne 2004; Peshin 2002).

Due to their increasingly extensive use, pesticide market and its related technology suffered a great change since 1940, becoming a very sophisticated industry that substituted the old pesticides by hundreds of agrochemicals highly effective and specialized, most of them derived from synthesis chemistry. The first products were based on simple organic compounds, as said, some of them showed high toxicity to mammals and intolerable risks for manufacturers and farmers. For this reason, the efforts of the industry were not only focused on the more effectiveness or specificity, but also to find harmless substances for humans.

The 1970's and 80's saw the introduction of the world's greatest selling herbicide, gluphosate, the low use rate sulfonylurea and imidazolinone herbicides. For insecticides the synthesis of a 3rd generation of pyrethroids took place, together with the introduction of avermectins, benzoyl ureas and Bacilus thuringiensis as a spray treatment. Many of the agrochemicals introduced at this time had a single mode of action, thus making them more selective, problems with resistance occurred and management strategies were introduce to combat this negative effect.

In the 1990's research activities concentrated on finding new members of existing families which have greater selectivity and better environmental and toxicological profiles. In addition neew families of agrochemicals have been introduce to the market such as the triazolopyrimidine, triketone and isoxazole herbicides , the strobilurin and azolone fungicides and chloronicotinyl, spinosyn, fiprole and diacylhydrazine insecticides. Table 3 summarizes the launching years of some common fitosanitary products since WWII, in the case of methomyl, imidacloprid, fosetyl, and the pesticides related to 2000's, the year matches its first registration date in US Environmental Protection Agency (US Environmental Protection Agency 2012).

It should be pointed out that except for captan, mecoprop, glyphosphate, diflubenzuron, imidacloprid, azoxystorbin, methomyl and fosetyl-Al, the listed pesticides, ranging from 1939 to the ends of 1980's, are nowadays all excluded from the list of approved sunstances for crop protection by the European Comission and its used is banned in any of the State Members (World Health Organization 2009). In the case of the products registered in the last decade, most of them are approved, while a certain amount are pending for approval (Dimethyl-disulfide) and the rest have no requested to be accepted in EU yet.

Table 2. Common pesticides and their launching year. Modified from (Matthews 2006) according to (US Environmental Protection Agency 2012; World Health Organization 2009).

Year	**Type**	**Pesticide**	**Year**	**Type**	**Pesticide**
1939	Insecticide	DDT	1971	Herbicide	glyphosphate
1942	Herbicide	2,4-D	1972	Insecticide	diflubenzuron
1943	Fungicide	zineb	1973	Insecticide	permethrin
1944	Insecticide	HCH (lindane)	1986	Fungicide	fosetyl-Al
1946	Insecticide	parathion	1990's	Insecticide	imidacloprid (1993)
1948	Insecticide	aldrin, dieldrin		Fungicide	azoxystorbin
1949	Fungicide	captan		Insecticide	Spinosad
1952	Insecticide	diazonon		Insecticide	tetrachlorvinphos
1953	Herbicide	mecoprop	2000's	Insecticide	gamma cyhalotrin
1955	Herbicide	paraquat		Herbicide	mesosulfuron-methyl
1956	Insecticide	carbaryl		Fungicide	fluopicolide
1965	Nematicide	aldicarb		Fungicide	etoxazole
1968	Fungicide	benomyl	2010	Herbicide	fimethyl-sulfide
1968	Insecticide	Methomyl		Nematicide	

New chemistry allowed better resistance management and improved selectivity. During the 90's mature products were refined in terms of use patterns with the introduction of newer and more user-friendly and environmentally safe formulations. While older products no longer covered by patents have moved to generic companies, those investing in R&D have not only diversified into Genetic Modified crops, but have also realized that the registration authorities are unlikely to accept the most toxic pesticides, nor those which are very present in the environment. The ideas about their use and indispensability are changing with the concept of integrated pest management (broad based ecological approach to pest control that integrates minimized pesticides/herbicides into a management system incorporating a range of observation, prevention and biological control practices) and pest resistant crops. These new trends impulse the use of averting tactics, biological regulations, etc. in order to discourage the development of pest populations and reduce the use of pesticides and other interventions to levels that are economically justified, and have contributed to reduce pesticide use (OECD 1999; OECD 1999). However the omnipresence of pesticides is still patent all around the world. In 2012, according to IPPC (International Plant Protection Convention), global sales of pesticides for plant pest control run around $45 billion per year (Food and Agriculture Organization of the United Nations 2012a). Almost 50% of this amount is due to herbicides sales, 25% to insecticides, 20% to fungicides and the remainder to other products. Most pesticides are used in North America, which accounts for about one-third, with Western Europe and East Asia accounting for the one-fifth each, and Latin America the next largest market.

1.4.1. ENVIRONMENTAL FATE OF PESTICIDES

Auxiliary agricultural products, such as fertilizers, pesticides, etc., can be blamed for a major impact on the environment when they are discharged to the environment. In the case of pesticides, they are considered a major concern due to the fact that most of them are biorecalcitrant substances with a relatively high average life (Hayasaka et al. 2012), that hardly degrade in natural ecosystems. Even its safe use can lead to loss of biodiversity, due to the fact that still today, most pesticides are not specifically targeting the pest only and during their application they also affect non-target plants and animals (Pesticide Action Network 2010).

Their presence has been detected in air, water and soil, and at all trophic levels, from plankton to large mammals, given that these compounds tend to bioaccumulate in many living beings. This increases their toxic and polluting potential as they move through food chains (Baranowska et al. 2005; Hayasaka et al. 2012; Pérez-Ruzafa et al. 2000; Wendt-Rasch et al. 2004).Traces of them can be found in almost every food commodity, surface and groundwater streams and sometimes even in natural fiber clothes. Endocrine disrupting effects, hepatic dysfunctions and reproductive disorders have been reported in literature (Bretveld et al. 2008; Cecchi et al. 2012) since the early 1970’s (Clement and Okey 1974). In intensive farming effluents the concentration of pesticides may reach considerably high levels (up to several hundred $mg{\cdot}L^{-1}$) and the methods conventionally used in sewage treatment plants, as filtration or secondary reactors, etc., are insufficient (Malato et al. 2001).

If the product is rationally and carefully applied to the recipient crops, it can still be affected by different processes such adsorption, transfer and degradation, depending on different properties of the media and the pesticide itself; processes that can take place separately, successively or simultaneously and causes pesticide distribution into four media: atmosphere, water, soil or biota, as represented in fig. 8.

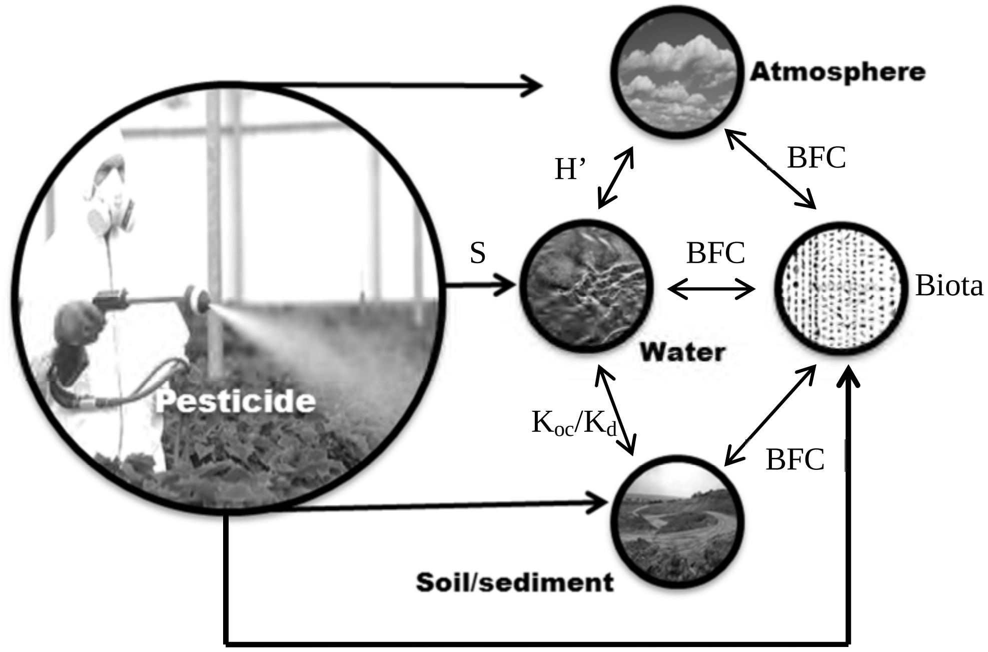


Figure 8: Diagrammatic representation of environmental fate of pesticides. Above the arrows, the principal propierties of the pesticides that determines its flow towards that media are represented by their initials. S, solubility, H', Henry's law constant, BCF, bioaccumulation factor, Koc, octanol/water partition coefficient, Kd, sorption coefficient.

1.4.2. PESTICIDE LEGISLATION

Regarding to pesticides legislation in European Union, the Commission updated the legislation in 2009, upgrading the previous Regulation 79/117/EEC and the Directives 94/414/EEC and 2006/42/EC. With the advice of its Directorate-General for Food and Health, in 2009 the EC published four pieces of legislation which made up the European Thematic Strategy for Pesticides (Commision of the European Communities 2002).

It consists of two directives, 2009/127/EC and 2009/128/EC, (European Parliament and Council 2009b; European Parliament and Council 2009c), and two regulations 1107/2009/ECC and 1185/2009/ECC (European Parliament and Council 2009a). Directive 2009/128/EC, establishing a framework for community action to achieve the sustainable use of pesticides, is the first piece of UE legislation governing the use of pesticides. It has been coming into force in a number of stages from 2011 to 2020 and its key feature is a requirement for Member States to develop National Action Plans designed to reduce further the risks associated with the use of pesticides and promote the low input systems (in particular, Integrated Pest Management, IPM), although some of the proposed measures are already being used as part of the existing national ranges of statutory and voluntary controls in some Member States (Stark 2011). In the case of the two Regulations which came into force in June 2011, aimed to increase the level of protection given to human health, animal welfare and the environment; and simplify procedures and offer a more even choice of products to farmers and growers across the

EU. Key elements which strengthen the current regime include establishing a system under which similar products could be compared and the more hazardous removed from the market, and introducing cut-off criteria designed to exclude the most hazardous compounds (Stark 2011), in order to perpetuate the work done since 1993 to 2009 with the review of existing pesticides that were on the market before1993.

This initiative, detailed in the Factsheet EU Action on Pesticides, "our food has become greener" (Directorate-General for Health and Consumers, European Commission 2009) reviewed all active substances used in plant protection within the EU. Each substance had to be evaluated as to whether it could be used safely with respect to human health (including residues in the food chain) and environment, in particular groundwater and non-target organisms (while EU legislate over the active principles, each State Member legislate about the commercial formulations of this principle used in its territory, always undergoing the European Legislation (Unsworth 2010). The approval system counted with the collaboration of individual State Members acting as rapporteurs to transmit its preliminary conclusions about the substance, while it was the responsibility of the industries to provide the data containing enough information for the safety evaluation. Of some 1000 analyzed active substances only 250 passed the EU safety assessment. There is a complete and free access database for consulting the approved or non-approved status of all the substances that have been ever registered in any of the Member States (Directorate-General for Health and Consumers, European Commission 2013).

The named data base also contains information about the Maximum Residue Levels of every active substance or commercial formulation tolerated by EU. These levels are based on the Regulation 396/2005/EC (European Parliament and Council 2005). Its guidelines, applicable to every Member State, harmonized and simplified pesticide MRL-regime for better consumer protection throughout the EU. The former rules were too complex as it combined consistent EU and divergent national rules. This situation led to confusion about which MRL was applicable. Beside it established a default MRL of 0.01 $mg{\cdot}kg^{-1}$ (set as a limit of determination) to those new commodities (being launched to the market everyday) where no specific MRL is set, unless a different default level is agreed, or until such time as an MRL is set on the basis of the evaluation of data, assuring the safety even of those products not analyzed included in the MRL-regime yet. Table 4 contains some of the already listed pesticides MRL for different food commodities.

Regarding to Spain, the first Spanish legislation on pesticides appeared just a year after the beginning of democracy, as a transposition of Council Directive 78/631/EEC, subsequently amended later and was mainly regulated by Royal Decree 3349/1983, which approved Health Technical Regulations for the manufacture, marketing and use of pesticides and; Royal Decree 162/1991, amended the previous RD.

Table 3 Some examples of MRL's for some of the pesticides in table 1.Its noticeable how high are the limits for fosetyl-Al indicating its low toxicity.

(mg/kg)	Fruit Fresh (melon)	Tree nuts (almonds)	Curcubits (cucumber)	Milk (cow)	Chicken
captan	0.02	0.3	0.1	0.02	0.02
benomyl	0.7	0.1	0.1	0.05	0.05
diflubenzuron	1	0.1	0.05	0.05	0.05
etoxazole	0.1	0.02	0.05	0.01	0.01
methomyl	0.02	0.02	0.02	0.02	0.02
imidacloprid	1	0.05	0.5	0.1	0.05
fosetyl-Al	75	2	75	0.1	0.1

In 1999 the European Parliament and Council published a new Directive, 1999/45/EC to homogenize the Member States regulations relating to the classification, packaging and labeling of dangerous preparations. However, regarding to the marketing of such products Royal Decree 2163/1994 already implemented the Community harmonized authorization to trade and using pesticides, so there was no need for amendments. 1994 Royal Decree also anticipated EC regulation and directives of 2009, especially in terms of sustainable use of pesticides, promotion of Integrated Pest Management, and protocols for machinery inspection. However, as Member State, if any aspect of European legislation overcomes national regulations, the law of reference is always the one dictated by EC.

Pesticides are still needed nowadays, especially for its use in large areas of major crop to cover the development costs. Even sustainable agriculture could require the use of these substances in a judicious way. Chemical technology could still work on safer substances in the frame of Integrated Pest Management. However the extreme position of official organisms are promoting the diminishment of investment by companies in more sophisticated substances, more selective and less toxic, due to the worry of wasting vast quantities of money in R&D in the case that the Governments decide not to approve even this more evolved compounds.

1.4.3. CARBAMATES INSECTICIDES

Carbamates were introduced into the market by Geigy Chemical Company in Switzerland in 1951 (Ware 2000). N-methyl carbamates, together with the organophosphorated and organochlorinated insecticides, belong to what is known as 2nd generation pesticides due to the year range in which they were developed (first decades after WWII). Among this generation, carbamates and organophosphorated pesticides are the most used due to be relatively safer for the environment tan organochlorinated. They present a lower half life, though their toxicity is noticeably high (Pisani et al. 2008).

Carbamates are derivatives of carbamic acid –NH(CO)OH–, an unestable substance, and are also related to the toxic alcaloid *physostigmine* isolated from calabar (*physostigma venenosum)*, originary from Nigeria.

The entry of carbamates to the organic systems can occur by ingestion, inhalation or contact with eyes, skin or other mucosae, either by direct contact or by proximity to contaminated media. In the case of superior animals, they are hydrolyzed enzymatically by the liver; degradation products are excreted by the kidneys and the liver (Reigart and Roberts 1999). Like organophosphorated, the mode of action of the carbamate is that of reversible inhibition of the vital enzyme cholinesterase (ChE). This inhibition is called cholinergic effect and manifests about an hour after exposure. The enzyme suffers a carbamylation of the active site, although this bind is more ephemeral than in the case of phosphorylation produced by organophosphorated (Padilla et al. 2007). In fact, nowadays, the use of carbamates is encouraged in opposition to the phosphoresced, especially the double N-methyled carbamates, for their lower potential to dramatically affect non-target species (Ware 2000).

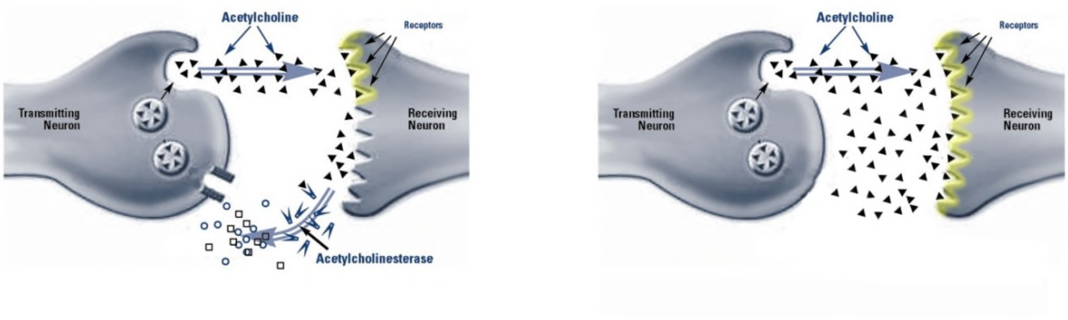


Figure 9. Normal and abnormal functioning of a cholinergic synapse. Typically cholinesterase, in its acetyled specie (AChE), efficiently hydrolyzes acetylcholine (Ach), released by the presynaptic terminal after the transmission is finished. The generated choline is then transported back into the presynaptic terminal, in order to be again acetyled and ready for the next use. When sufficient cholinesterase inhibitors bind to ChE, acetylcholine degradation is hindered and it accumulates in the synaptic cleft. What leads to persistent stimulation of the cholinergic receptors on the postsynaptic cell. (Caption and figure adapted from (Pope et al. 2005)). The cholinesterase inhibitors avoid the generation of this lysing enzyme, a high amount of acetylcholine is free in the postsynaptic media, being susceptible to bind the receptors provoking the constant stimulus of the synapse.

The cholinergic effect prevents the degradation of neurotransmissor acetylcholine, which in normal conditions is caused by cholinesterase, hydrolyzing it into choline and acetic acid, as represented in fig. 9. Animals with low levels of ChE present symptoms of anorexia, lethargy, and psychological and behavioral disorders. This is the due to the accumulation of acetylcholine in their synaptic media, which provokes the continuous stimulation of muscles, glands, and the central nervous system itself. The cholinergic neuron is unable to return to its resting state and is kept continuously activated (Pope et al. 2005). All these symptoms diminish noticeably their survival and reproductive capabilities (Pisani et al. 2008).

METHOMYL

The pesticides involved in this work to simulate hydroponics effluents were chosen as part of the range of products used in the pilot greenhouse of the CENIT-MEDIODIA program, and it was convenient to focus the work in already employed substances. One of the principal reasons for which it was chosen as a target compound to study were is moderate persistence in environment (half-life of 14 days in soils (Univ. California-Davis et al.)) together with its high mobility in the cropping media (US Environmental Protection Agency 1998). This is consistent with the fact that methomyl has been detected in ground water and can also contaminate surface water as a result of spray drift during application or by runoff from treated system, what is susceptible to happen in the case of traditional agriculture, but also in semi closed hydroponics systems if the effluent are not properly treated .

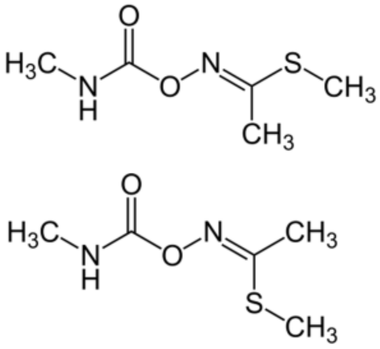


Figure 10. Molecular structure of methomyl, S-methyl-N((methulcarbamoyl)oxy)thioactimidate.

Methomyl is an N-methylated species of the family of the carbamates. In its pure state and environmental conditions, it is a white crystalline solid with slight sulfur odor. Fig. 10 represents its molecular structure. It was firstly registered in the United States in October 1968 by E. I. du Pont de Nemours & Company Inc. (US Environmental Protection Agency 1998). It is still used as a broad-spectrum insecticide, but is particularly suitable for Lepidoptera, Coleopterons and Dipterous for its cholinergic effect; however it is also applied as ovicide against cotton bollworms and budworms. Methomyl is registered on a wide variety of sites including field, vegetable and orchard crops: turf; livestock quarters; commercial premises; and refuse containers (there are no homeowner uses of methomyl). In the case of Spanish greenhouses, to the date of this project methomyl is sprayed on crops of tomatoes, lettuce and some ornamental plants, although it is also valid in the case of other vegetables, citrus, snuff, cotton, herbs, etc. Table 4 summarizes its main characteristics and properties.

Methomyl is an effective contact and systemic pesticide, which can affect the target insects either by dermal exposure, inhalation, ingestion or absorption by their mucosal membranes. It has the advantage over other carbamates that it can be absorbed by the plant without causing phytotoxicity, turning it into a source of poison.

As a carbamate, methomyl acts by inhibiting cholinesterase, an enzyme essential for the proper functioning of the nervous system. Mammals, including humans are also susceptible to being poisoning due to this insecticide. Its highest toxicity is achieved when ingested or absorbed through the eyes, the inhalation can cause a moderate poisoning, and dermal exposure causes only slight toxicity. The harshness of the symptoms ranges from those of a common cold, headache, tearing, discomfort, to severe nervous system collapse and death. However, the carbamates are generally excreted easily and do not accumulate in tissues. Thus, in moderate poisoning, on cessation of exposure, cholinesterase easily returns to normal levels. Except in cases of fatal poisoning, the symptoms do not extend beyond 24 hours.

Table 4. Main properties of methomyl. (Agriculture & Environment Research Unit (AERU), University of Hertfordshire 2009; Kesley et al. 2011)

Common name	Methomyl
Chemical name	S-methyl-N((methylcarbamoyl)oxy) thioactimidate
CASS RN.	16752-77-5
CEE N.	240-815-0
OMS N.	1196
Stoichiometric structure	$C_5H_{10}N_2O_2S$
Molecular weight	162.2
Solubility, water 25ºC (g·L^{-1})	57.9
Solubility, ethanol 25ºC (g·kg^{-1})	420
Melting point (ºC)	77
Vapor pressure, 25ºC (mPa)	0.72
Lethal DoseLD$_{50}$, birds (mg·kg^{-1})	24.2
Lethal Dose LD$_{50}$, rats (mg·kg^{-1})	30
Lethal Dose LD$_{50}$, fishes 96 h(mg·L^{-1})	0.9
Persistence and degradability	Stable in water during 30 d (pH 5-7, 25ºC); DT$_{50}$ *c.* 30 d (pH 9, 25ºC). Stable over 140ºC. Estable to solar light, 120 d exposition.

According to World Health Organization (WHO) classification of pesticides (World Health Organization 2009), which categorization is summarized in table 5, methomyl is considered class Ib, highly hazardous. Nevertheless in 2012 is still approved and used in US; although the Environmental Protection Agency included it in a document scheduling the registration review of a number of chemical in order to reassess its safety between 2012 and 2015 (US Environmental Protection Agency 2012). In Europe, its use was reapproved by current legislation Directive 2009/115/EC and Regulation 540/2011/ECC, after being included in the suitable list of Directive 91/414/EC and excluded from it temporarily in 2010. In the case of Spain, it becomes extensive the approval of the European Union. The expiration date of the European approval is 31/08/2019 (Directorate-General for Health and Consumers, European Commission 2013).

Table 5. WHO Classification. Based on LD$_{50}$ for the rat (mg/kg body weight).*The terms *solids* and *liquids* refer to the physical state of the product or formulation being classified.

		Oral Toxicity#		Dermal Toxicity#	
Class	**Hazard Level**	**Solids***	**Liquids***	**Solids***	**Liquids***
Ia	Extremely hazardous	>5	>20	<10	<40
Ib	Highly hazardous	5-50	20-200	10-100	40-400
II	Moderately hazardous	50-500	200-2000	100-1000	400-4000
III	Slightly hazardous	>500	>2000	>1000	>4000

U	Unclassified

The product used in this work is a commercial formulation of methomyl provided by Aragonesas Agro S. A. called Tomilo 20L. In it, the active ingredient is concentrated to 200 mg·L^{-1} in 82 % of dimethyl sulfoxide, an aprotic solvent, which makes solubilizes the active ingredient and makes the formula more volatile to facilitate its absorption by the insect. It is recommended to dissolve in water and apply dispersed in the leaves, for tomato, eggplant, broccoli, citrus, pome and stone fruit, snuff, cotton, beets, hops, and ornamental grasses and ornamental nurseries.

The neonicotinoid insecticides, part of the group of organochlorines, are the only substances from this family that are still accepted in Europe and USA (Reigart and Roberts 1999). The development of this class of insecticides began with work in the 1980s by Shell and the 1990s by Bayer (Yamamoto and Casida 1999). Their low toxicity to non-target organisms including users and manufacturers, together with their high efficacy at low dosages (Matthews 2006) entitled them as 3rd generation pesticides. They are the most important insecticides introduced to global market since the synthetic pyrethroids. Today, neonicotinoids are registered globally in more than 120 countries and found to be effective against sucking pests such as aphids, leafhoppers, planthoppers, thrips, whiteflies, etc.

These insecticides are similar and modeled after natural nicotine, historically employed as bioinsecticide. In the early 1980's, industry paid its attention in this bioplaguicides, especially in nicotine, trying to emulate its effects but improving its stability and specificity, and lowering its toxicity to mammals. After unsuccessful attempts of producing synthetic relatives to nicotine ended up in photo-unstable species lead to the introduction of a 3-pyridymethyl group on nicotinic nitro-methylene heterocycles, remarkably improving the insecticidal activity. Further optimization resulted in the invention of imidacloprid in 1990, which is reasonably persistent. Several analogs of imidacloprid followed. These commercial products were first baptized as chloronicotinyl species, however because there were compounds having no chloropyridyl group, and based on the mode of action proposed the term "neonicotinoids" to cover all these compounds (Yamamoto and Casida 1999). Nowadays, neonicotinoids, noticeably imidacloprid, are widely used around the world especially where insects are resistant to the earlier types of insecticide (Matthews 2006).

1.4.4. NEONICOTINOID INSECTICIDES

The neonicotinoid insecticides, part of the group of organochlorines, are the only substances from this family that are still accepted in Europe and USA (Reigart and Roberts 1999). The development of this class of insecticides began with work in the 1980s by Shell and the 1990s by Bayer (Yamamoto and Casida 1999). Their low toxicity to non-target organisms including users and manufacturers, together with their

high efficacy at low dosages (Matthews 2006) entitled them as 3rd generation pesticides. They are the most important insecticides introduced to global market since the synthetic pyrethroids. Today, neonicotinoids are registered globally in more than 120 countries and found to be effective against sucking pests such as aphids, leafhoppers, planthoppers, thrips, whiteflies, etc.

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Like carbamates and organophosphorated insecticides, neonicotinoids effect is focused in the nervous system, at the cholinergic synapse, but it a different mode action. Neonicotinoids are selective agonists of the insect nicotinic acetylcholine receptors (nAChRs0) (Shao et al. 2011) and bind them irreversibly, thanks to the fact that nicotinic group mimics Ach, they both have similar dimensions and relative distribution of charge and polarity. These binding blocks permanently the receptor at the same time it keeps it constantly active leading paralysis which leads to death (Matthews 2006; Ware 2000).

Because the neonicotinoids block a specific neural pathway that is more abundant in insects than warm-blooded animals, these insecticides are selectively more toxic to insects than mammals, however neonicotinoids can be absorbed from the gut and also by the lungs and across the skin. They are excreted biliary, although, as organochlorined they produce urinary metabolites that can harm the excretive system. The endocrine system may be also altered by continuous contact with neonicotinoids, which may alter the reproductive development, inhibit lactation, etc.(Reigart and Roberts 1999)

In the last years, a special concern is growing related to the harmful effects of neonicotinoids over honey bees colonies near established the application sites (Badiou-Bénéteau et al. 2012; Gregorc et al.; Iwasa et al. 2004). This concern ended up with the banning of three neonicotinoids (imidacloprid, clotianidin and tiametoxam) by the European Food Safety Authority (EFSA) while this thesis was being written (Doncel and Sevillano 2013), based on the report "EFSA identifies risks to bees from neonicotinoids" (EFSA, European Panel of Food Safety Authority 2013).

IMIDACLOPRID

As methomyl, imidacloprid was part of the range of products used in the pilot greenhouse of the CENIT-MEDIODIA program. This neonicotinoid is currently the most widely used insecticide in the world. Although it is now off patent, the primary manufacturer of this chemical is Bayer CropScience, (part of Bayer AG). Between 1986 and 1988 the first patent was filled and granted for imidacloprid in US by a Japanesse Company (Shiokawa et al. 1988). However, between 1992 and 1993 Miles, Inc. (later Bayer CropScience) applied for registration of imidacloprid for turfgrass and ornamentals in the United States, and in 1994, the U.S. Environmental Protection Agency approved the first registration of imidacloprid as eligible to be used in US (US Environmental Protection Agency 2012). Fig. 11 represents the molecular formulation of this neonicotinoid.

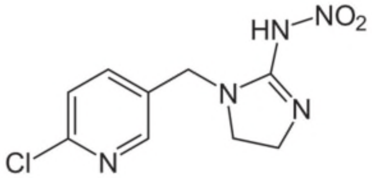


Figure 11. Molecular structure of imidacloprid, N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide

Table 6 comprises some of the characteristics that make this neonicotinoid susceptible of being part of this study. The low K_{oc} (soil adsorption coefficient) of 132 to 310, combined with high water solubility, suggest a potential to leach to ground water and to enter streams and ponds via drift during application or in runoff water (Hayasaka et al. 2012). However, the moderate K_{ow} (octonal-water coefficient) of 3.7, combined with its rapid photodegradation in water (half-time t1/2 < 3 h) and on soil ($t_{50\%}$39 days) suggest a low potential for bioaccumulation (Bacey 2001).

Table 6. Main properties of imidacloprid. (Bacey 2001; National Pesticide Information Center 2010)

Common name	Imidacloprid
Chemical name	N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide
CASS RN.	13826-41-3
Stoichiometric structure	$C_9H_{10}ClN_5O$
Molecular weight	255.7
Solubility, water 20ºC ($g{\cdot}L^{-1}$)	0.51
Solubility, isopropanol 20ºC ($g{\cdot}L^{-1}$)	1.0-2.0
Melting point (ºC)	136.4-143.8
Vapor pressure, 20ºC (mPa)	200
Lethal Dose LD_{50}, rats (ingested) ($mg{\cdot}kg^{-1}$)	450
Lethal Dose LD_{50}, rainbow trouts (ingested) ($mg{\cdot}L^{-1}$)	211
Octanol-Water partition coef. Log K_{ow}, 21ºC	0.57
Soil Sorption coef. K_d	156-960, mean values 249-336
Persistence and degradability	Low vapor pressure and Henry's law coefficient indicates low volatility from water, is unlikely to disperse in air over a large area. Hydrolysis of imidacloprid can range from 33 to 44 days at pH 7 and 25oC. The aqueous photolysis half-life is less than 3 hours.

Like most members of its family, imidacloprid is a systemic insecticide. It has good root-systemic characteristics and notable contact and stomach (ingestion) action. It acts on the central nervous system causing irreversible blockage of postsynaptic nicotinergic acetylcholine receptor (Ware 2000). Imidacloprid is usually applied directly to the seeds, or to the plants substrates from where it is absorbed by the roots and circulates through the xylem along the plant (Byrne and Toscano 2006; Lagalante and Greenbacker 2007; Mota-Sanchez et al. 2009). With this mode of application and action, consistency and durability is given to the pest controlling effect, thus preventing further outbreaks. Besides, the application on leaves enable the neonicotinoid to penetrate the layers and battle the pest from the leaf lower surface (Elbert et al. 2008). It is used with cotton, rice, cereals, peanuts, potatoes, vegetables, pecans and turf, for the control of sucking insects, soil insects, whiteflies, termites, turf insects, with long residual control.

World Health Organization (WHO) categorizes imidacloprid as, moderately hazardous, II (World Health Organization 2009). In fact like methomyl is also candidate for EPA revision of its acceptance in US by 2015 (US Environmental Protection Agency 2012). In Europe, it was approved under Reg. (EC) No 1107/2009 and 540/2011, and Directive

2008/116. It was approved by 08/01/2009 and its expiration date is 07/01/2019 (Directorate-General for Health and Consumers, European Commission 2013).

The commercial formulation employed in this project was Kohinor, from Aragonesas Agro S.A. The active ingredient is present in a concentration of 200 $mg \cdot L^{-1}$, together with ethanol and dimethyl esters as inert ingredients. It is recommended to be used pulverized as in foliar application. The manufacturer indicates its suitability over tomato, pepper, eggplant, cucurbits, broccoli and cauliflower, lettuce, cabbage, beans, pome fruit, peach and nectarine, apricot, cherry and plum, almond, hazelnut, hops, citrus, olives, vine, potato, and big ornamentals.

1.4.5. ORGANOPHOSPHATE FUNGICIDES AND FOSETYL-AL

Fungicidal action is usually expressed in one of two physically visible ways: the inhibition of spore germination or the inhibition of fungus growth. Most fungicides kill the spore immediately following germination, or even prevent the very germination, as organophosphates fungicides do. This newer systemic fungicides have eradicant properties and are able also to stop the progress of existing infections (Ware 2000). They can be used directly over crops, but also over seeds and stored products to prevent from spoilt.

Organophosphates like iprobenfos, toclofos-methyl, pyrazophos, isoprothiolane and edifenphos, fosetyl-Al are systemic fungicides able to be absorbed and translocated throughout the plant. Besides, in general, fungicidal effect is due to a metabolic inhibitor action. Like other fungicide families such as inorganic coopper and mercury compounds, dithiocarbamates, thiadiazoles, etc., organophosphates act as inhibitors of critical enzimes for the reproduction of the fungal organism by their denaturalization through different paths, mainly inactivating enzimatical vital groups like –SH, –SR or –NH_2. As a family, organophosphates are effective against peronospirales, rice blast, powdery mildews and dreschlera (Chaube and Pundhir 2005).

However, as mentioned for neonicotinoids and carbamates, organophosphorate fungicides are also susceptible to cause cholinergic effect (with more intensity than organocholinated substances) affecting the nervous system of non-target organisms, even of human beings. This main drawback makes it prescriptive to handle these substances with extreme precaution in their concentrated form (Carille 1995).

FOSETYL-AL

Fosetyl-Al structure is depicted in fig. 12. As can be seen it is the aluminum salt of phosphonic acid monoethyl ester.

Fosetyl-Al was first registered in US in 1986 (US Environmental Protection Agency 2012), although it was manufactured in 1977 for the first time by Bayer CropScience. In Europe it was reapproved in 2007 (with an expiration date of this approval for 2017), and nowadays it is authorized in all Member States (Directorate-General for Health and Consumers, European Commission 2013). By now, it is not classified under The WHO Recommended Classification of Pesticides by Hazard (World Health Organization 2009).

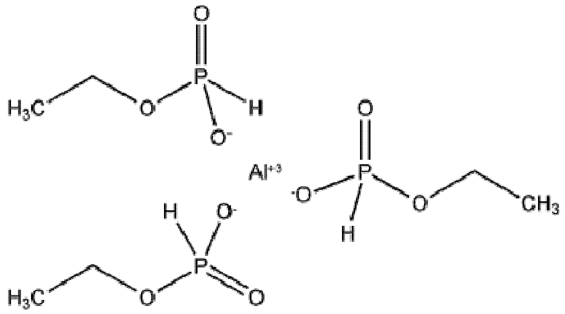


Figure 12: Molecular structure of fosetyl-Al, *aluminium tris-O-ethylphosphonate*

This pesticide is produced by the reaction of aluminum nitrate in aqueous solution of ethyl phosphite or by reaction of sodium ethyl phosphite and aluminum trioxide at 60 °C. It is an odorless white powdery-crystalline solid, which melts at 215 °C and has a very low vapor pressure. As can be seen among its main properties in table 7, it is poorly soluble in most organic solvents, with exception of methanol. Fosetyl-Al is fairly soluble in water but it dissolves rather slowly and, following dissociation of the salt, the liberated ions undergo various reactions (Food and Agriculture Organization of the United Nations 2011). This product is highly effective against root, stem and foliage diseases caused by oomycetes (mainly root-attacking phytophtora and downy mildews) in a variety of crops. Oomycetes are not sctrictly fungi; they comprise a distinct phylogenetic lineage of fungus-like eukaryotic microorganisms. However, as the use the same mechanisms as fungi to infect plants, the products devoted to its control are also called fungicides.

Regarding to its way of action, fosetyl-Al has two properties that makes it unique in front of other fungicides even those from organophosphates family. On one hand, in opposition to most of these products which, once inside the plant, are translocated upwards the transpiration stream, fosetyl-Al is carried also downwards the circulating system (Carille 1995). It makes this product optimum for the treatment of roots and stem diseases by means of foliar application. On the other hand, fosetyl-Al has been lately reported to stimulate auto-defense reactions in the crops, by the synthesis of phytoalexines against oomycetes (Agnios 2005). These are antimicrobial substances synthesized de *novo* by plants that accumulate rapidly at areas susceptible of pathogen infection. They are broad spectrum inhibitors and are chemically diverse with different types characteristic of particular plant species (Favaron et al. 2009). The mechanism through what fosetyl-Al is able to induce the production of this substances is largely unknown, but there are evidences that it depends both in the very plant and the environmental conditions (Andreu et al. 2006) and is probably the derived phosphite group the specie to blame.

Table 7. Fosetyl-Al main properties. (Agriculture & Environment Research Unit (AERU), University of Hertfordshire 2009; Kesley et al. 2011)

Common name	Fosetyl
Chemical name	Aluminum tris(O-ethylphosphonate)
CASS RN.	39148-24-8
Stoichiometric structure	$C_6H_{18}O_9P_3$-Al
Molecular weight	354.1
Solubility, water 20ºC (g·L^{-1})	120
Solubility, methanol 20ºC (g·L^{-1})	0.92
Melting point (ºC)	215
Vapor pressure, 20ºC (mPa)	Mineral salt, not applicable
Persistence and degradability	Fosetyl is stable under dry conditions for a minimum of 3 months (50ºC)

Regarding to its way of action, fosetyl-Al has two properties that makes it unique in front of other fungicides even those from organophosphates family. On one hand, in opposition to most of these products which, once inside the plant, are translocated upwards the transpiration stream, fosetyl-Al is carried also downwards the circulating system (Carille 1995). It makes this product optimum for the treatment of roots and stem diseases by means of foliar application. On the other hand, fosetyl-Al has been lately reported to stimulate auto-defense reactions in the crops, by the synthesis of phytoalexines against oomycetes (Agnios 2005). These are antimicrobial substances synthesized de *novo* by plants that accumulate rapidly at areas susceptible of pathogen infection. They are broad spectrum inhibitors and are chemically diverse with different types characteristic of particular plant species (Favaron et al. 2009). The mechanism through what fosetyl-Al is able to induce the production of this substances is largely unknown, but there are evidences that it depends both in the very plant and the environmental conditions (Andreu et al. 2006) and is probably the derived phosphite group the specie to blame.

As said, due to the capacity of inhibiting fungal-like spore germination and the indirect stimulation of the plants natural defense mechanisms, fosetyl-Al can be considered relatively long-lasting product and resistance rarely occurs (Food and Agriculture Organization of the United Nations 2011). According to this, although this fungicide is quite traditional, its newly discovered properties justified its use even in sophisticated agricultural system such as advanced greenhouses from project CENIT-MEDIDODIA.

The commercial formulation employed in this study is Fosbel-80, wetable powder, from Probelte S.A., with an 80% w/w of active principle, accompanied with inert inorganic ingredients such as kaolin. Its use is indicated for seed orchards, avocado and strawberries, citrus, cupressaceae, curcubitaceae, hops, turf, spinachs and similar.

1.5. ADVANCED OXIDATION PROCESSES

Domestic use and industrial activity, of especially impact among the developed countries, generate high amounts of residual wastewater, whose direct disposal to natural channels causes considerable harm to the environment. Since the beginning of the 20th Century, health concerns, which were later joined also by environmental concerns, the construction to more or less sophisticated wastewater treatment facilities was encouraged, where physical and biological processes were the main available treatments, even nowadays. However, industrial activity, intensive agriculture, domestic use of manufactured commodities, etc., have been releasing to the environment, mainly through outlet water streams, different kinds of non-biodegradable compounds. These substances, which range from drugs and personal care products to pesticides, dyes, etc., have been revealed as refractory to traditional treatments. In the last decades, public and politic opinion have paid special attention to this issue, mainly since the release of the European Directive 2000/60/CE (European Parliament and Council 2000) that stressed the need to adopt measures against water pollution in order to achieve a progressive reduction of pollutants (Pera-Titus et al. 2004). For this reason, more sophisticated processes had to be implemented to provide a final treatment stage to further improve the effluent quality before it is discharged to the receiving environment.

Late 1980’s and 1990’s saw a growing interest in a family of processes called Advanced Oxidation Processes for the treatment of drinking water and wastewater (Glaze et al. 1987; Glaze et al. 1995). They consists on a set of chemical procedures designed to remediate water pollution based on the oxidation potential of in-situ generated hydroxyl radicals (·OH). The interest on these technologies is still increasing in our days, as can be seen in fig.13, which depicts the increasing attention on this subject by the number of entries related to AOPs included in Science Direct database since 1994.

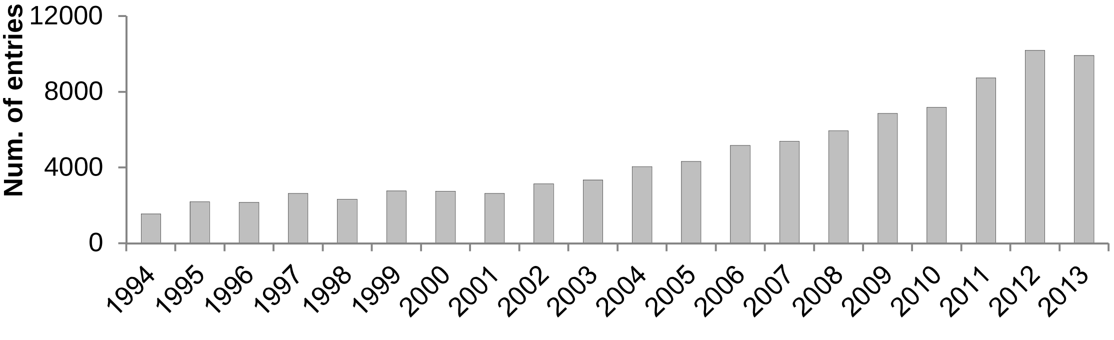


Figure 13. Number of entries searching “Advanced Oxidation Processes” and selecting just Journal publications in Science Direct. Survey performed at July 1^{st}, 2013.

1.5.1. HYDROXYL RADICAL

Hydroxyl radical is a powerful non-selective chemical oxidant, which has strong oxidation potential and acts through a rapid kinetic with most organic compounds. It has a short life time, 70 ns in aqueous media (Land and Ebert 1967). Due to the high reactivity of these radical species in driving oxidation processes, AOPs have been proven as suitable for achieving partial to complete abatement and mineralization of different kinds of pollutants, hopefully through even less toxic species (Malato et al. 2002).

Table 8 Standard oxidation potentials against Standard Hydrogen Electrode (SHE) of some oxidants in acidic media. Adapted from (Beltrán et al. 1997a), except for *, from (Hunsberger 1977).

Oxidant	**E° (V)**
Fluorine	3.03
Hydroxyl radical	2.80
Atomic oxygen	2.42*
Ozone	2.07
Hydrogen peroxide	1.77
Potassium permanganate	1.67
Hypobromous acid	1.59
Chlorine dioxide	1.50*
Hypochlorous acid	1.49
Chlorine	1.36
Oxygen	1.20*
Bromine	1.09

Table 8 summarizes some reduction potentials of well-known oxidants species in acidic media. Among them the high potential of the hydroxyl radical stands out over most of the substances'. ·OH lack of selectivity when attacking organic matter makes it significantly more useful than other compounds, especially in water treatment, where changing substrates are common and most of the organic content to oxidize is susceptible of react with this kind of oxidant.

According to Pignatello et al., (Pignatello et al. 2006), the hydroxyl radical promotes oxidation through three different pathways:

- Hydrogen atom abstraction from C-H, N-H or U-H bonds. This reaction takes place typical in the presence of alkanes and alcohols.

$$\cdot OH + R - H \rightarrow H_2O + R \cdot \qquad \text{r. 1}$$

- Electrophile addition to a C=C double bond, or to an aromatic ring, hydroxylation. It takes places in the presence of alkenes and aromatic compounds.

$$\cdot OH + = \rightarrow HO - C \cdot \qquad \text{r. 2}$$

- Electronic transference to ·OH radical, although this path happens rarely with organic compounds

The way that hydroxyl radical oxidize organic matter consists on a series of chain reactions that generated organics radicals that keep their degradation process until it ultimately became CO_2 and H_2O (reactions 3 to 7). They could also get reduced or polymerize, as can be seen in reaction 8.

$$\cdot OH + R_tH \rightarrow H_2O + R_t \cdot \qquad \text{r. 3}$$

$$R_t \cdot + H_2O_2 \rightarrow ROH + HO \cdot \qquad \text{r. 4}$$

$$R_t \cdot + O_2 \rightarrow ROO \cdot \qquad \text{r. 5}$$

$$R_t \cdot + Fe^{3+} \rightarrow R^+ + Fe^{2+} \qquad \text{r. 6}$$

$$R_t \cdot + Fe^{2+} \rightarrow R^- + Fe^{3+} \qquad \text{r. 7}$$

$$2R_t \cdot \rightarrow Dimers \qquad \text{r. 8}$$

Aromatic molecules with electron donor groups (-OH, $-NH_2$, etc.) react faster than those with electron acceptor groups ($-NO_2$, -COOH, etc.) (Sirés 2006).

There are certain organic substances that cannot be oxidized by hydroxyl radical, mainly short chain molecules such as acetic, maleic, and oxalic acid, acetone or simple organochlorine species, i. e. chloroform or tetrachloroethane (Bigda 1995). Other substances like organic salts can act consuming the radicals to the detriment of the organic load, these are called *scavengers*. Even and excess of H_2O_2 could also act as hydroxyl scavenger, producing the formation of hydroperoxyl radical ($\cdot O_2H$), that has a much lower reactivity than ·OH (Legrini et al. 1993).

1.5.2. AOPS CLASSIFICATION

AOPs can be classified depending on the source of the oxidizing species or the method employed for its production. A commonly used classification is shown in table 9, while some details of these techniques are given bellow.

According to (Suty et al. 2004) the decision on the most appropriate AOP to use must take the following factors into consideration:

- The nature, physic-chemical properties and concentration of the pollutants to be removed
- The biodegradability of the pollutant
- The presence of hydroxyl radical scavengers and compounds that absorb UV radiation
- The content of suspended material
- The existing content of a catalyst in the effluent
- The suitability of handling sludge, the possibility of heating the effluent and controlling pH

The cost of the system is obviously an important factor but is very often not taken into account in many application studies.

Table 9. Advanced Oxidation process according to the source of hydroxyl radical.

Advanced Oxidation Processes	Photolysis	• UV Photolysis • V-UV Photolysis
	Ozone based AOP	• Ozonation/ alcaline conditions • Ozonatio + UV and/or H_2O_2 • Ozonation+ catalizer
	H_2O_2 based AOP	• Fenton • Fenton-like • Photo-Fenton • H_2O_2/UV • Electro-Fenton
	Thermal AOP	• Super Critical Wet Oxidation • Wet oxidation • Wet Oxidation + H_2O_2
	High energy AOP	• Ultrasound technologies • Electrochemical Oxidation • Electron beam technologies • Microwaves enhances processes
	Photocatalysis	

Photolysis

This treatment does not need the addition of an external chemical. The oxidation of the target compounds takes place through two different paths based on the interaction between the media and light radiation with wavelengths comprised between 140-200 nm. On one hand, the direct action of UV light over the organic matter itself could bring about its dissociation into smaller fragments. On the other, the incidence of radiation over the water molecules, and other susceptible substrates, produces hydroxyl radicals that contribute to the degradation of the organic pollutants in the system (Gonzalez et al. 2004). In the case of (Benitez et al. 2013), this technique is used for the degradation of emerging contaminants. The effect of the AOP over the toxicity of the effluent is also observed in this study.

The main drawbacks of this technology are the energy consumption of the light source and the harmful UV radiation employed in the process.

Ozone based AOPs

Ozonation chemistry is complex; it is characterized by driving the oxidation through two mechanisms; the direct reaction with the dissolved molecular ozone (O_3) and the indirect reactions with the radical species ($\cdot OH$, $\cdot O_2H$), that are formed when ozone decomposes in water (Hoigné and Bader 1975; Hoigné and Bader 1976). Which is the predominant pathway will depend on the nature of the target compound, the pH of the media and the ozone concentration (Andreozzi et al. 1991). Like in the case of photolysis, ozone treatment may be enhanced by the addition of hydrogen peroxide and/or UV radiation as an extra source of active hydro-radicals (Beltrán et al. 1997b).

The advantage on working with this technology is that when bubbled into water, free ozone quickly decomposes to oxygen, harmless to environment, so it is like no external chemical is added to the media. However, the main drawback of this technique is the handling of ozone gas that can easily degrade installations materials in case of escape, it can cause moderate to severe harm in airways, can generate explosive atmospheres, and contributes to the greenhouse effect at tropospheric level.

Hydrogen peroxide based AOPs

H_2O_2 is a safe, efficient and easy to use chemical oxidant suitable for a wide usage on contamination prevention. However, since hydrogen peroxide itself is not an excellent oxidant, it has to be combined with other substances, such as metal salts or ozone, or needs a certain radiation dosage, to produce the desired degradation results by its conversions into more radicals.

Among these techniques, UV/H_2O_2 process, the Fenton process, its UV light empowered version and all related processes are worth to be mentioned. While Fenton and photo-Fenton will be detailed described in subsequent sections, a brief description of UV/H_2O_2 would say that it is a process which involves the generation of $\cdot OH$ radical through photolysis of H_2O_2 caused by 254 nm UV radiation (Afzal et al. 2012; Ghaly et al. 2001).

Hot Advanced Oxidation Processes

These processes differ from the rest of the AOPs not only in terms of operating conditions but also in the concentration of the pollutants present in the wastewater. Among these technologies Supercritical Water Oxidation (SCWO), Subcritical Oxidation or Wet Oxidation (WO) and Wet Peroxide Oxidation (WPO) can be mentioned. In opposition to other AOPs, hot processes are used mainly for highly concentrated wastewaters, and are based on the use of high operational pressures and temperatures, that lead to the production of free radicals by water hydrolysis. For example SCWO takes place above water critical point (T>375 ºC and P>22.1 MPa) (García-Molina et al. 2005).

Photocatalysis

This AOP counts on the acceleration of a photoreaction by the presence of a catalyst. In this reaction, light is absorbed by the catalyst depending on its photocatalytic activity that relies on its ability to create electron-hole pairs. In the case of water treatment photocatalysis is based on the use of TiO_2 or other solid semiconductors as heterogeneous catalyzer. Illuminated semiconductor particles could catalyze a wide range of redox reactions of organic and inorganic substrates (Fujishima et al. 2007).

The principle of this methodology involves the activation of the semiconductor by artificial or sunlight. A semiconductor is characterized by valence and conduction bands, and the area between them is the band gap. The absorption of photons with energy higher than the band gap energy results in the promotion of an electron from the valence to the conduction band, with a concomitant hole generation in the valence band (Andreozzi et al. 1999). This promote the generation of hydroxyl radicals and other radical species that contribute to organic matter oxidation.

TiO_2 was used to improve the biodegradability of an effluent polluted with dimethoate by Arques et al., (Arques et al. 2007). It resulted on an efficient pre-treatment to deal with aqueous solutions of the pesticide. Different biodegradability tests indicated that complete removal of dimethoate was necessary to detoxify the solution.

High energy AOPs: Ultrasounds, microwave enhanced processes and electron beam technologies

The basis of ultrasonically induced oxidation is the generation of cavitation bubbles. Each bubble acts as a reactor with supercritical operating conditions. In aqueous systems at an ultrasonic frequency of 20 kHz a cavitation bubble is supposed to reach 4000 K of temperature and 1000 bar of pressure.

Microwaves lie in the region of the electromagnetic spectrum between millimeter waves and radio waves (frequencies of between 30 and 0.3GHz). This relatively new technique (beginning of 2000s) was implemented for the release of nutrients and the reduction of solids from sewage sludge. The microwave heating process could break down particles, resulting in carbon and other nutrients becoming solubilized and the sludge content reduced (Guiquing 2008).

Ultrasound and microwaves have been reported as useful for water decontamination (Horikoshi et al. 2011), surface cleaning, soil washing, control of air-borne contamination, or sewage treatment (Remya and Lin 2011).

The electron beam process uses the Coulomb interaction between accelerated electrons and atoms or molecules of gases, liquids or solids. By these interaction ions, thermalized electrons, excited states and radicals are formed. Generated free radicals react with organic matter (Cleland et al. 1984).

1.5.3. AOPS FOR WATER REUSE

The capability of AOPs to eliminate hazardous pollutants that scape from secondary treatments and to inactivate microbes is stated in several works related to reclaimed water reuse that apply this technology as a way to ensure quality standards.

According to (Khoufi et al. 2006; Khoufi et al. 2009) oil-mill water could be adapted to agricultural reuse through electro-Fenton process. (Ince and Tezcanh 1999)tested Fenton, Fenton-like reactions, together with their photo-empowered alternatives for detoxifying textile dye-bath effluents to resend the flow to the tannery cycle. The same idea presented (Zhang et al. 2011) that experimented the efficiency of ozonation and electrofloculation of the removal of polluting substances from textile industry wastewater. This study also stated, through toxicity analysis, the convenience of the use of O_3 dosing in front of other conventional tertiary techniques.

Ozonation is precisely one of the AOPs that have raised more interest in recent times. (Rodríguez et al. 2012) essayed it for the elimination of emerging pollutants such as herbicides, antibiotics, and β-blockers, among others. The oxidant power of O_3 seems to be also efficient in combination with slow sand filtration for reaching German standards for reuse from wastewater treatment plant effluents according to (Hübner et al. 2012). Included in a train of treatments, ozonation showed its capability to reduce the level of non-specific toxicity of a secondary effluent polluted with pharmaceutical substances (Reungoat et al. 2010). A wide survey of over seventy individual pollutants (mainly pharmaceuticals and personal care products) that transpose conventional urban secondary treatments was performed by Rosal and colleagues. (Allen 1997; Rosal et al. 2009; Rosal et al. 2010). Their study proved that ozonation allowed the removal of many individual pollutants, and permitted the determination of kinetic constant for some of the studied substances.

(Rosario-Ortiz et al. 2010) also worked on pharmaceuticals depletion, in this case, by means of UV/H_2O_2. (Pisarenko et al. 2012) evaluated an ozone and ozone/peroxide oxidation process for trace organic contaminants in water reuse application, stating the potential formation of NDMA, N-Nitrosodimethylamine, a harmful by-product of oxidation that tend to be generated during oxidation in reuse applications. As this substance is toxic and suspected human carcinogen, its occurrence has to be prevented by any means in water reuse, therefore toxicity evaluations need to be carried out. Like the ones performed by (Zhang et al. 2011)that leaded to advise to add a time of soil aquifer treatment to reduce potential toxicity generated by the AOP itself.

(Chong et al. 2012) focused on the evaluation of the technical, economic and environmental feasibility for applying AOPs at decentralized WWTP. (Hermosilla et al. 2012; Klamerth et al. 2009; Westerhoff et al. 2009) studied TiO_2 oxidative capabilities;

while Klamerth and Hermosilla focused on the chemical reaction itself, Westerhoff worked on the coupling of heterogeneous catalysis with a biological reactor. (Muñoz et al. 2009) used Life Cycle Assessment to compare different scenarios involving wastewater reuse, with special focus on toxicity-related impact categories. Its results showed that wastewater reuse after applying ozonation in combination with hydrogen peroxide appears to be the best choice from an ecotoxicity perspective.

(Meneses et al. 2010) also worked with Life Cycle Assesment, but in this case applied to AOP for disinfection. Traditional disinfection methods, such as chlorination and ultraviolet treatment, were compared to ozonation and O_3/H_2O_2, to evaluate these techniques and to assess the environmental advantages and drawbacks of urban wastewater reuse in non-potable applications (both agricultural and urban uses). Meneses and colleagues concluded that ozonation and ozonation plus hydrogen peroxide disinfection technologies have similar environmental profiles, and achieved 50% higher values for disinfection parameters in comparison to the traditional systems. (Rodríguez-Chueca et al. 2012) also studied Fenton and photo-Fenton processes for disinfection. In this case, the main objectives were to model and optimize their performance by means of experimental design. Similarly (García-Fernández et al. 2012) assessed photo-Fenton microbes inactivation potential; García-Fernandez and colleagues compared it to its solar empowered version and other AOPs. The following gradation could be determined according to the disinfecting effectiveness of each process: photo-Fenton> solar photo-Fenton> H_2O_2/sunlight>Fe^{3+}/sunlight. (Rojas-Valencia et al. 2011) proposed ozonation biological inactivation for reuse in agriculture and landscape irrigation.

More integrating works were presented by (Blanco et al. 2012; Feng et al. 2010). They both tried to optimize Fenton parameters to obtain better results in chemical oxidation and disinfection, in the first case to resend certain effluents to a tannery cycle under Chinese regulations; in the second case, to meet quality standards according to RD1620/2007.

1.5.4. FENTON

Fenton reaction generally occurs in chemical and biological systems as well as in the natural environment. It has been known for more than a century (Fenton 1894). Fenton stated that ferrous ion is capable of activate the hydrogen peroxide deriving in the formation of strong oxidizing species. He observed the oxidation of tartaric acid by H_2O_2 in the presence of ferrous iron ions. Nevertheless, its application as an oxidizing process for degrading hazardous organics was not referred in literature until the late 1960s (Huang et al. 1993; Neyens and Baeyens 2003). However, Fenton is still currently accepted as one of the most effective methods for the oxidation of organic pollutants, including in wastewater and other wastes (Barbusiński 2009). Table 10 summarizes some practical cases of Fenton reaction applied to reduce COD from different origin effluents.

As mentioned, classic Fenton theory explains the oxidation of a wide range of organic molecules by the interaction between hydrogen peroxide and Fe^{2+} catalyzer, usually soluble in water, by means of a series of free radical chain reactions. However, other theorist proposed that iron ions aquo-complexes actually caused the decomposition of the peroxide into hydroxyl radicals (Pignatello 1992). There is still a third way of thinking suggested by Bossmann, (Bossmann et al. 1998), that postulates the participation in the process of hypervalent iron species (Fe^{4+}, Fe^{5+}).

As can be seen, the nature of the species taking part in the reaction is still under discussion and it has been a subject of controversy in the past ad recent Fenton oxidation related literature (Bossmann et al. 1998; Gogate and Pandit 2004; Pignatello et al. 1999). Among these references, two reaction pathways for the first step of Fenton chemistry have been stand up: a radical pathway, which considers ·OH radical production, and a non-radical pathway considering ferryl ion production as the most reactive specie (Deguillaume et al. 2005). Considering the fact that Fenton reaction is common in artificial and natural systems where conditions may be diverse, it is highly probable that there is more than one universal reaction mechanism (Barbusiński 2009).

Table 10. Use of the Fenton reagent (H_2O_2/Fe^{2+}) to reduce COD in wastewater (Suty et al. 2004).

Industry	**Q (m^3/d)**	**COD_0 (mg/L)**	**Treatment cond.**	**COD_f (mg/L)**	**Site**	**Quantity of sludge discharged**
Abrasive paper washing water	10-20	10000	Batch T = 50ºC $FeSO_4$	900	Main treatment	80t/y 45% TS
De-inked pulp newspaper process water	50000	200	Continuous No heating FeC_{l2}	<100	Tertiary Treatment	On site incineration
Chemical specialties process wastewater	20	9000	Batch T=80ºC 2bar FeSO4	1600	Main treatment	30t/y 50%TS
MMA and PMMA process wastewater	3000	800-1000	Continuous No heating $FeSO_4$	<500	Main treatment	ND
Textile industry process wastewater	100	>2500	Continuous T=100ºC 3bar $FeSO_4$	500	Pre-Treatment	ND
Explosive process wastewater	200	Up to 10^{-5} (10 g/L Cl-)	Continuous T=100ºC 3bar Mixture Mn^{n+}	<100	Main treatment	70t/y 45%TS

This thesis works in particular around the hypothetical mechanism proposed by Barb and colleagues (Barb et al. 1951a; Barb et al. 1951b; Barb et al. 1949) consisting in hydrogen peroxide decomposition, in acidic pH, in the dark and in the absence of organic compounds, according to the following sequence of the reactions that comprise active radicals formation:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH$ r. 9

$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \cdot O_2H + H^+$ r. 10

$\cdot OH + H_2O_2 \rightarrow \cdot O_2H + H_2O$ r. 11

$\cdot OH + Fe^{2+} \rightarrow Fe^{3+} + OH^-$ r. 12

$Fe^{3+} + \cdot O_2H \rightarrow Fe^{2+} + \cdot O_2 + H^+$ r. 13

$Fe^{2+} + \cdot O_2H + H^+ \rightarrow Fe^{3+} + H_2O_2$ r. 14

$\cdot O_2H + \cdot O_2H \rightarrow H_2O_s + O_2$ r. 15

$\cdot O_2H + H_2O_2 \rightarrow \cdot OH + H_2O + O_2$ r. 16

This mechanism can be considered a modification of the original mechanism from Haber and Weiss, who suggested in 1932 that ·OH production by one-electron reduction of H_2O_2 by Fe^{2+} (Haber,F.,Weiss,J. 1934). As can be seen, hydroxyl radicals are generated in reaction 9 and 16. Reaction 11 consumes hydrogen peroxide in detriment of hydroxyl radicals; nevertheless this reaction is several orders of magnitude slower than reaction 9.

The efficiency of the Fenton reaction depends mainly on H_2O_2 concentration, Fe^{2+}/H_2O_2 ratio, pH and reaction time. The initial concentration of the pollutant and its character as well as temperature, also have a substantial influence on the final efficiency. The reagents concentration is indeed especially important due to the fact that an excess of them could cause counterproductive increase in the kinetics of undesirable reactions. For example, an excess of H_2O_2 produces a deceleration of a few orders of magnitude in the process, due to too rapid oxidation of Fe^{2+} to Fe^{3+}. This could also increase the formation of hydroperoxyl radicals according to reaction 11, scavenging the hydroxyl radicals jeopardizing the oxidation of the hypothetical organic load. Some authors claim that the molar ratio H_2O_2/Fe used in water treatment should be between 100 and 1000 (González 2009).

Inorganic species such as Cl^-, SO_4^{2-}, $H_2PO_4^-/HPO_4^{2-}$, etc., could affect also the global reaction rates of Fenton process as has been reported in previous literature. They can cause three different effects: i) complexing reactions with Fe2+ and Fe3+ (reducing the photoactivity of the system (Pignatello et al. 2006)); ii) precipitating reactions that reduce dissolved active Fe(III); iii) hydroxyl radicals scavenging that favors the formation of other inorganic radicals less reactive (Bacardit et al. 2007; Lipczynska-Kochany et al. 1995; Pignatello et al. 2006) and subsequent oxidation reactions with those species.

PHOTO-FENTON

This is an interesting variation of Fenton in which radiation of a certain wavelength (180 to 410 nm) (Wadley and Waite 2004), even visible spectrum (Oliveros et al. 1997), contribute to the *dark* Fenton, increasing its efficiency . Photo-Fenton reaction leads generally to higher velocities of degradation and mineralization, due to the creation of an extra path for hydroxyl radical formation thanks to the photoreduction of Fe^{3+} to Fe^{2+}, according to reaction 17.

$$Fe^{2+} + H_2O + h\nu \rightarrow Fe^{2+} + H^+ + \cdot OH \qquad \text{r. 17}$$

In this way, ferrous cation is continuously recycled thanks to radiation, and there is always certain amount of Fe^{2+} available in the media (Zepp et al. 1992). Additionally, according to Pignatello also the formation and photodegradation of Fe^{3+} aquo-complexes (predominant in acidic conditions) contribute to the generation of hydroxyl radicals (Pignatello et al. 2006) with 180-410 nm radiation.

$$Fe^{2+} + H_2O_2 \rightarrow Fe(OH)^{2+} + \cdot OH \qquad \text{r. 18}$$

$$Fe(OH)^{2+} + h\nu \rightarrow Fe^{2+} + \cdot OH \qquad \text{r. 19}$$

pH is a parameter that could have a negative influence over Fenton process (Kavitha and Palanivelu 2004). Generally it is accepted that optimum pH for Fenton reagents is 2.8 (Pignatello 1992). At this pH Fe^{3+} ion coexist with its complex $Fe(OH)^{2+}$ in a ratio of 1:1, both are the most photoactive species of the system. At lower pH, the concentration of $Fe(OH)^{2+}$ decreases, favoring the generation of other complexes less photoactive. Over pH 2.8, Fe^{3+} precipitates as $Fe(OH)_3$.

PHOTO-FENTON REACTION APPLIED TO PESTICIDE POLLUTED WATERS,

STATE OF THE ART

As has been already mentioned, pesticides cause a major impact on the environment due to its characteristics as biorecalcitrant and bioaccumulative (Hayasaka et al. 2012). These facts raise the need, on the first hand, to avoid every uncontrolled dumping, and on second hand, to promote the optimal use and reuse of polluted effluent. Furthermore, because of the great potential of contamination by pesticides themselves and their residues (Cardeal et al. 2011), it is also advisable to treat these sources of diffuse pollution "in-situ", to achieve standards that allow water to be returned to its cycle, as part of public sewage services or returning to agricultural irrigation systems. The particular characteristic of these substances make necessary to use specific techniques for their treatment, which could range from activated carbon or membrane filtration, specialized biological processes or even a combination of them. On one hand, the maintenance and survival of a consortium of specialized microorganisms could be time and money-consuming, requires qualified personnel, and every fluctuation in the feed could destabilize the bioreactor and even collapse it. On the other hand, filtration procedures do not eliminate the pollutant rather concentrate it in another effluent that should be treated in the end.

Advanced Oxidation Processes are presented as an interesting alternative based on a chemical oxidation treatment that aims to get rid of the pesticide by its complete mineralization (conversion into H_2O and CO_2) or at least by its degradation into less toxic and biodegradable species. Numerous authors have published interesting works on pesticides degradation by these promising technologies, in many cases, coupled with biological treatments. In these references, not only the extent of contaminant removal according to the treatment conditions is discussed, but also concepts such as toxicity and biodegradability before and after chemical treatment are evaluated, and how they affect the biological process associated.

A broad majority of these studies described in literature demonstrate a great efficiency of photo-Fenton in the decontamination of systems polluted by pesticides. Several factors influence the rate of degradation through this reaction: chemical structure of the pollutants, pH, iron concentration and its source, hydrogen peroxide, the organic load of the effluent and the nature of the light source.

Basic research about photo-Fenton applied to pesticide oxidation and AOP determine degradation kinetics enabling a further modeling. Since reaction mechanisms are complex for the photo catalytic degradation of organic compounds, a rigorous kinetic study cannot be performed (Saritha et al. 2007). However, works like Badawy et al. or Evgenidou et al.(Evgenidou et al. 2007), stated the adequacy of a pseudofirst order kinetic to explain photo empowered mineralization. According to their results, the oxidation rate is influenced by many factors such pH value, reaction time, COD:H_2O_2, Fe^{2+}:H_2O_2 ratios, the amount of iron salt and its valence, and even reaction temperature.

One of the main operating drawbacks of the photo-Fenton process is pH settings, which has to be lowered artificially to 3-2.5 (as at higher pH Fe^{2+} cations precipitates). This inconvenience has encouraged research centered on obtaining similar results at neutral pH in order to reduce operating costs (Bernabeu et al. 2012). As a consequence, recent studies have been focused on the use of different iron sources also in pesticide treatment field. In this way, stable iron chelates have been essayed, such as ferrioxalates (Colombo et al. 2011; Trovó et al. 2008) or citrates (Silva et al. 2007; Silva et al. 2010). Nonetheless, the use of chelates, which are usually a source of DOC, is not recommended when the process is applied as a tertiary treatment and treated water is discharged to the receiving environment. An option to address this problem is the use of supported iron catalyzers, such as zeolites (Gonzalez-Olmos et al. 2012) (phenol degradation) or other engineered doped materials (Gajovi et al. 2011). However, the need to remove the solid catalyzer from the effluent before its emission to the environment is a major disadvantage that ballasts this system. There is still an option to ensure the photo-Fenton performing at mild conditions a process settings that comprises iron dosage, instead of the total addition of the catalyzer at the beginning of the reaction (Carra et al. 2012).

Process optimization is frequently carried out by application of chemometric tools. Such strategies are generally more efficient and economical than the univariate approach, since they allow interactions between two or more variables to be studied within the context of influence of all other system parameters. This procedure has been applied several times to the study of photo-Fenton pesticide oxidation. The central composite design (CCD) and response surface methodology (RSM) were proposed to evaluate the influences of individual factors (H2O2 and Fe^{2+} concentrations) as well as their interactive influences in works from Micó and Colombo and colleagues (Colombo et al. 2013; Micó et al. 2010a), among others.

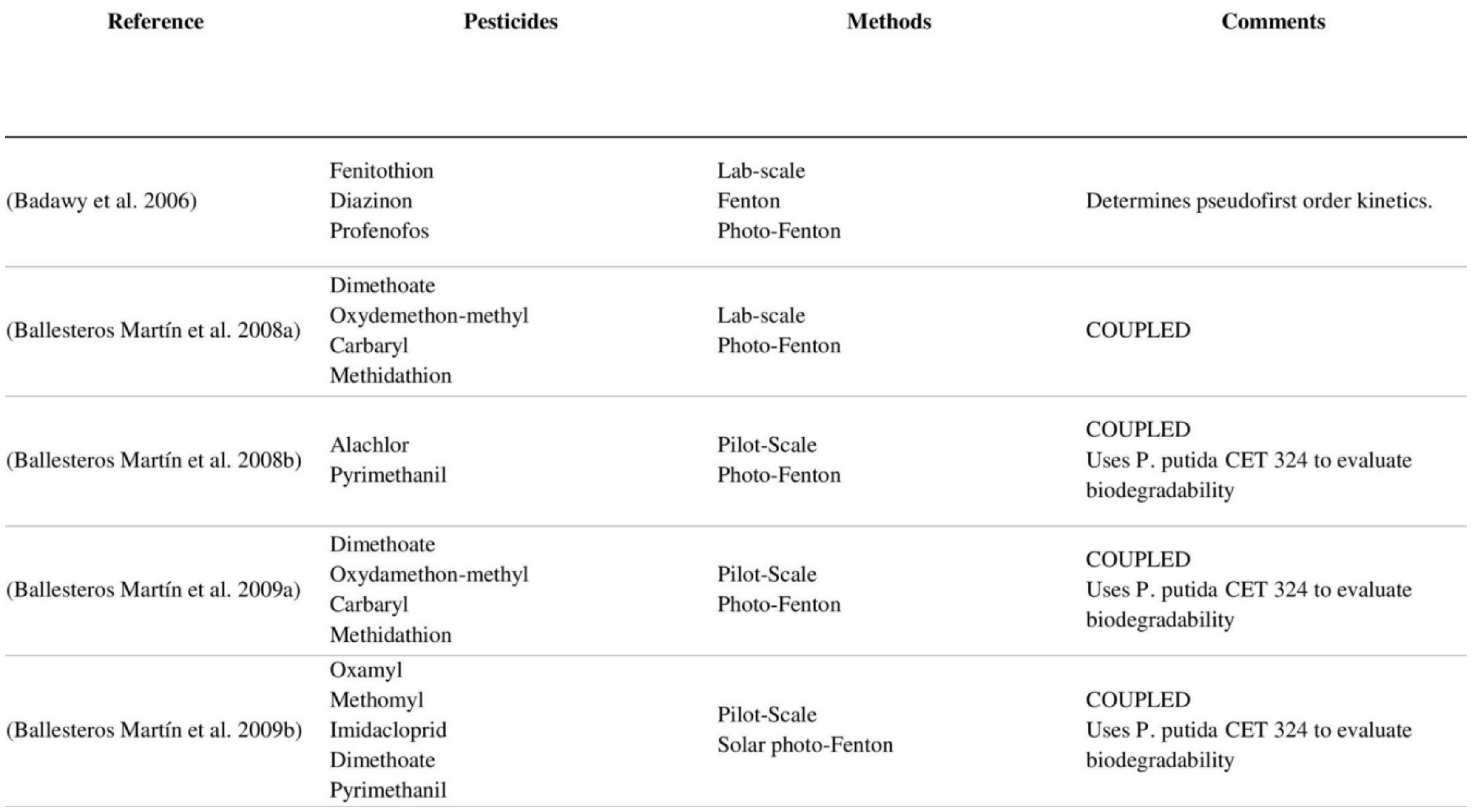
Several studies are focused in the comparison of different treatment techniques to degrade pesticide content. By instance, a work by Gozzi et al. (Gozzi et al. 2012)compares Fenton, photo-Fenton and ozonation technologies on the oxidation of chlorimuron-ethyl. Among its conclusions it highlights that the photo-Fenton system showed the best results when compared to the other studied systems, and can be considered an important technique for the treatment of wastewater containing this pesticide. In the case of 4-Chloro-2-Nitrophenol, a comparative assessment using various AOPs (UV, H2O2, UV/H2O2, Fenton, UV/Fenton and UV/TiO2) was performed by (Saritha et al. 2007). The study concluded again that the degradation of 4C-2-NP was strongly accelerated by the photochemical oxidation processes. It is photo-Fenton and its advantages which step over other photochemical treatment processes. Tamimi, Micó and colleagues (Micó et al. 2010b; Tamimi et al. 2008) both focused their comparative work on methomyl insecticide. The first one compared dark and enlightened photo-Fenton, and the results showed that Fenton and photo-Fenton processes are powerful methods for degradation of methomyl, but photo-Fenton reaction seemed to be is more efficient. In the second case, the comparison took place between photo-Fenton and ozonation processes. Micó also concluded that photo-Fenton was more efficient. Instead of comparing photo-Fenton with other systems, Farré et al. (Farré et al. 2007a) compared the efficiency of different photocatalytic processes combined with ozonation. Photo-Fenton/ozonation and Photocatalysis (TiO2)/ozonation lead to a rapid decrease of the concentration of the biorecalcitrant pesticides, alachlor, atrazine, chlorfenvinfos, diuron and PCP in aqueous solutions. However the best degradation results were obtained for the photo-Fenton combination

Microorganisms, especially bacteria, are considered to be the principal agents for the degradation of pesticides in bioremediation processes. Their presence in all environments around the world, even in agricultural soils, suggests that this microactivity could lead to an accelerated degradation on the pesticides. Many authors show the possibilities of combined photo-Fenton reaction, with an ulterior biological reactor,(Ballesteros Martín et al. 2008a; Ballesteros Martín et al. 2008b; Ballesteros Martín et al. 2009a; Ballesteros Martín et al. 2009b; Lapertot et al. 2007; Malato et al. 2007; Oller et al. 2007; Vilar et al. 2012; Zapata et al. 2010). In many cases, biodegradability measurements are performed previous to the integration of the process, such in the cases of Ballesteros et al., who base their values on the behavior of P. putida CET 324 to evaluate biodegradability. BOD_5/COD essays, together with vibrio fischeri toxicity analysis, were performed by Lapertot et al. to monitor biodegradability enhancement after photo-Fenton treatment (Lapertot et al. 2006).

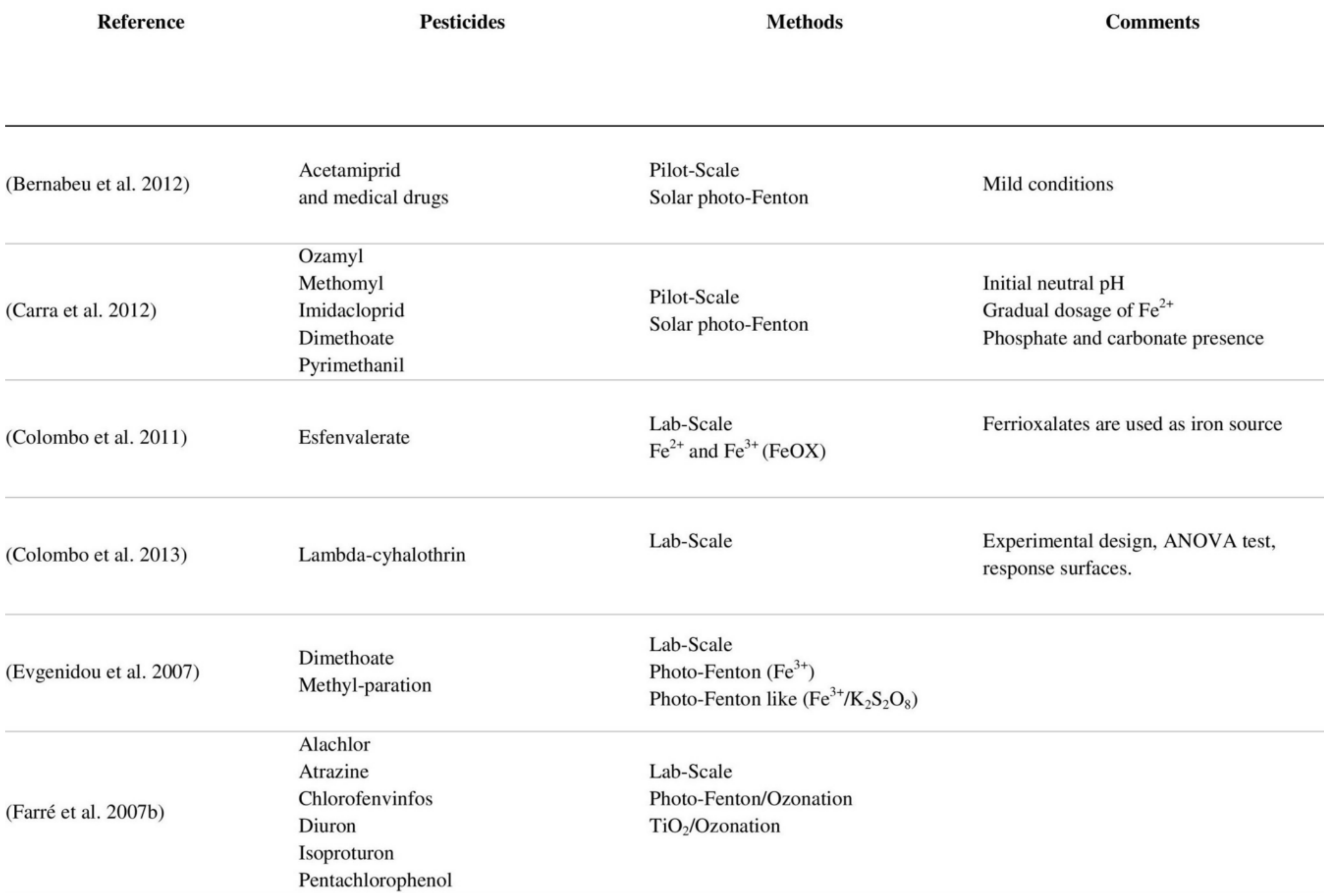
Many of the latest references work on the feasibility of using solar light as a source of radiation for improving the sustainability of the process (Maldonado et al. 2007) and even its efficiency. As it is considered a very promising possibility of cost reduction (Bauer and Fallmann 1997), different researching groups are working on the implementation of this technology in solar light irradiated pilot plants. This point of view has become popular especially in certain researching groups, mainly settled in Almería (Spain), (Universidad de Almería and Plataforma Solar de Almería-CIEMAT), (Ballesteros Martín et al. 2009b; Bernabeu et al. 2012; Carra et al. 2012; Hincapié et al. 2006; Jiménez et al. 2011; Lapertot et al. 2006; Lapertot et al. 2007; Maldonado et al. 2007; Oller et al. 2006; Oller et al. 2007); Alcoy (Spain) (Universitat Politècnica de València), (Soler et al. 2011); and Porto (Portugal), (Universidade do Porto), (Vilar et al. 2012).

Table 11 summarizes all these references and the main features of their studies.

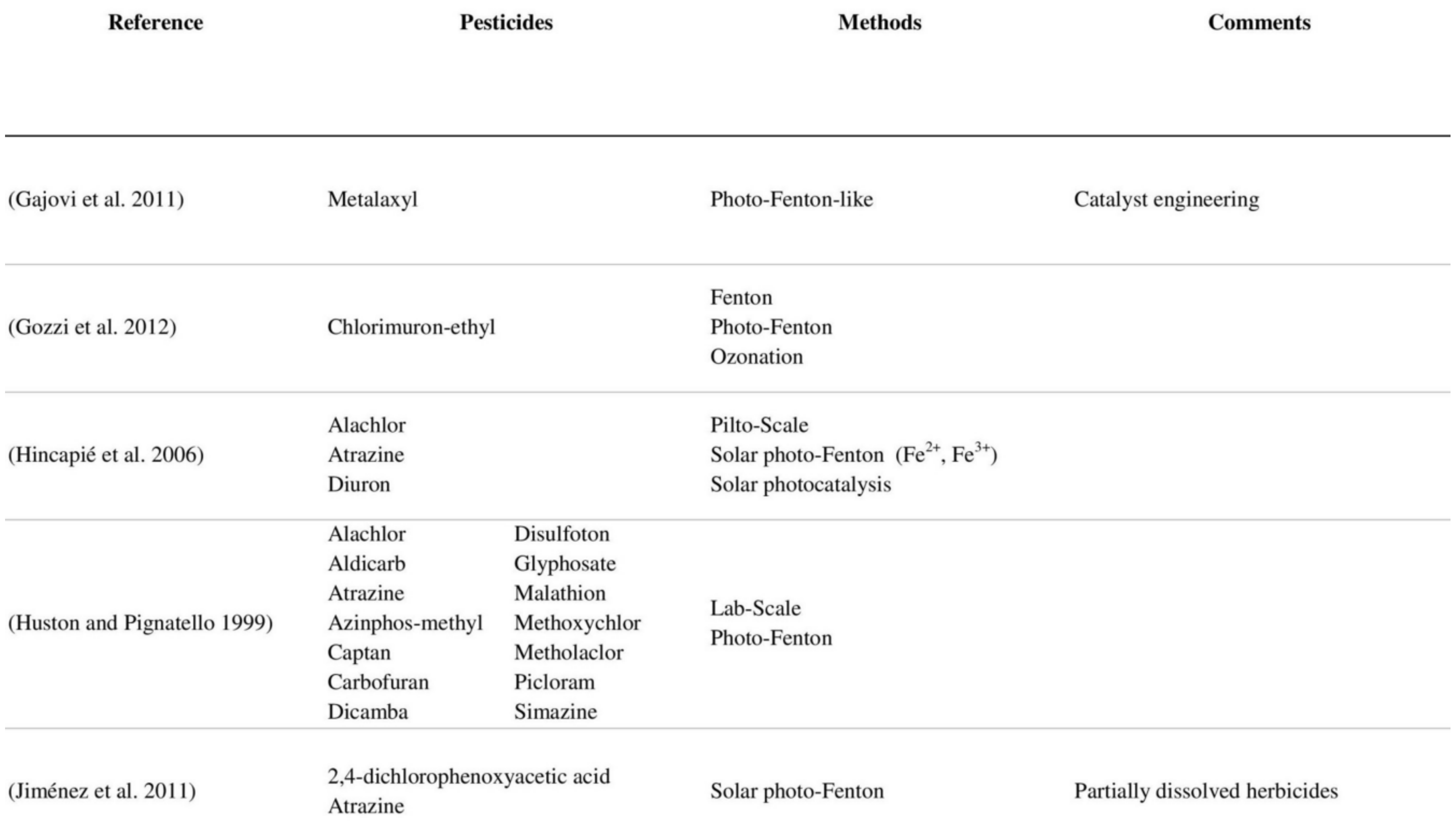
Table 11. Photo-Fenton reaction over pesticide polluted effluents, state of the art.



Reference	**Pesticides**	**Methods**	**Comments**
(Badawy et al. 2006)	Fenitothion Diazinon Profenofos	Lab-scale Fenton Photo-Fenton	Determines pseudofirst order kinetics.
(Ballesteros Martín et al. 2008a)	Dimethoate Oxydemethon-methyl Carbaryl Methidathion	Lab-scale Photo-Fenton	COUPLED
(Ballesteros Martín et al. 2008b)	Alachlor Pyrimethanil	Pilot-Scale Photo-Fenton	COUPLED Uses P. putida CET 324 to evaluate biodegradability
(Ballesteros Martín et al. 2009a)	Dimethoate Oxydamethon-methyl Carbaryl Methidathion	Pilot-Scale Photo-Fenton	COUPLED Uses P. putida CET 324 to evaluate biodegradability
(Ballesteros Martín et al. 2009b)	Oxamyl Methomyl Imidacloprid Dimethoate Pyrimethanil	Pilot-Scale Solar photo-Fenton	COUPLED Uses P. putida CET 324 to evaluate biodegradability



Reference	Pesticides	Methods	Comments
(Bernabeu et al. 2012)	Acetamiprid and medical drugs	Pilot-Scale Solar photo-Fenton	Mild conditions
(Carra et al. 2012)	Ozamyl Methomyl Imidacloprid Dimethoate Pyrimethanil	Pilot-Scale Solar photo-Fenton	Initial neutral pH Gradual dosage of Fe^{2+} Phosphate and carbonate presence
(Colombo et al. 2011)	Esfenvalerate	Lab-Scale Fe^{2+} and Fe^{3+} (FeOX)	Ferrioxalates are used as iron source
(Colombo et al. 2013)	Lambda-cyhalothrin	Lab-Scale	Experimental design, ANOVA test, response surfaces.
(Evgenidou et al. 2007)	Dimethoate Methyl-paration	Lab-Scale Photo-Fenton (Fe^{3+}) Photo-Fenton like ($Fe^{3+}/K_2S_2O_8$)	
(Farré et al. 2007b)	Alachlor Atrazine Chlorofenvinfos Diuron Isoproturon Pentachlorophenol	Lab-Scale Photo-Fenton/Ozonation TiO_2/Ozonation	



Reference	Pesticides	Methods	Comments
(Gajovi et al. 2011)	Metalaxyl	Photo-Fenton-like	Catalyst engineering
(Gozzi et al. 2012)	Chlorimuron-ethyl	Fenton Photo-Fenton Ozonation	
(Hincapié et al. 2006)	Alachlor Atrazine Diuron	Pilto-Scale Solar photo-Fenton (Fe^{2+}, Fe^{3+}) Solar photocatalysis	
(Huston and Pignatello 1999)	Alachlor Aldicarb Atrazine Azinphos-methyl Captan Carbofuran Dicamba Disulfoton Glyphosate Malathion Methoxychlor Metholaclor Picloram Simazine	Lab-Scale Photo-Fenton	
(Jiménez et al. 2011)	2,4-dichlorophenoxyacetic acid Atrazine	Solar photo-Fenton	Partially dissolved herbicides

Reference	Pesticides	Methods	Comments
(Lapertot et al. 2006)	Alachlor Atrazine Chlorfenvinphos Diuron Isoproturon	Pilot-Scale Solar photo-Fenton	Biodegradability and toxicity tests
(Lapertot et al. 2007)	Alachlor Atrazine Chlorofenviphos Diuron Isoproturon	Lab-Scale Photo-Fenton	COUPLED
(Malato et al. 2007)	MPG	Industrial-Scale Photo-Fenton	COUPLED
(Maldonado et al. 2007)	Alachlor Atrazine Chlorofenviphos Diuron Isoproturon Pentachlorophenol	Pilot-Scale Solar photo-Fenton Solar photocatalysis	
(Al Momani et al. 2007)	Vydine	Pilot-Scale Solar: UV Photolysis H_2O_2/UV Photo-Fenton	

Reference	Pesticides	Methods	Comments
(Navarro et al. 2011)	Atrazine Azoxystrobin Cyprodinil Fludioxonil Hexaconazole Kresosim-methyl Pyrimethanil Pririmicarb Propyzamide Tebuconazole Triadimenil	Pilot-Scale Photo-Fenton	Agriculture leaching water
(Oller et al. 2006)	Cymoxanil Methomyl Oxamyl Dimethoate Pyrimethanil Telone	Pilot-Scale Solar photocatalysis Solar photo-Fenton	
(Oller et al. 2007)	Cymoxanil Methomyl Oxamyl Dimethoate Pyrimethanil	Pilot-Scale Solar photocatalysis Solar photo-Fenton	COUPLED
(Saritha et al. 2007)	4-chloro-2-nitrophenol	Lab-Scale UV photolysis H_2O_2 UV/ H_2O_2 Fenton Photo –Fenton Photocatalysis	Determines pseudofirst kinetics
(Segura et al. 2008)	Imidacloprid	Lab-Scale Photo-Fenton	

Reference	**Pesticides**	**Methods**	**Comments**
(Silva et al. 2012)	Metalaxyl	Photo-Fenton	Highly concentrated pesticide COUPLED with lagooning (Solanum nigrum L. weed)
(Silva et al. 2007)	Tebuthiuron	Solar Photo-Fenton	Iron citrates
(Silva et al. 2010)	Tebuthiuron	Solar Photo-Fenton	Iron citrates Studies intermediates
(Sirtori et al. 2012)	Pyrimethanil	Pilot-scale Photo-Fenton	Study of chlorinated by-products Saline conditions $C_0(NaCl)= 5\ mg \cdot L^{-1}$
(Soler et al. 2011)	Methyl-oxydemethion Methidathion Carbaryl Dimethoate	Solar photo-Fenton	Presence of organic matter
(Tamimi et al. 2008)	Methomyl	Lab-Scale Fenton Photo-Fenton $UV+Fe^{2+}$ $UV+H_2O_2$	

Reference	Pesticides	Methods	Comments
(Trovó et al. 2008)	Terbuthiron Diuron 2,4-dichlorophenoxyacetic acid	Lab-Scale Photo-Fenton	Ferroxilates are used as iron source Uses multivariate analysis
(Vilar et al. 2012)	Benthazone S-methachlor Terbutylazine Atrazine Desisopropilatrazina Alachlor Pririmetanil Imidacloprid Isoproturon Tebuconazole Chlorotoluron Diuron Simazine Dimethoate Linuron Terbuthylazone-desethyl	Pilot-Scale Solar Photo-Fenton	COUPLED
(Zapata et al. 2010)	Oxamyl Dimethoate Imidacloprid Pyrimethanil	Scale-up Solar photo-Fenton	COUPLED

1.5.5. OZONATION

Ozone is a strong oxidant able to take part in many chemical reactions with organic and inorganic substances. While table 12 summarizes its main molecular properties, fig. 14 shows its bent structure as a triatomic molecule. Ozone can be structured as a resonance hybrid with a single bond on one side and double bond on the other producing an overall bond order of 1.5 for each side.

Table 12. Main molecular properties of ozone.

Molecular formula	O_3
Molecular weight (g·mol^{-1})	47.998
Density (g·L^{-1})	2.144 (at 0ºC, gas)
Melting point (ºC)	-192.5
Boiling point (ºC)	-111.9
Solubility in water (g·L^{-1})	0.0105
Std. enthalpy of formation (kJ·mol^{-1})	142.3
Std. molar enthalpy (kJ·mol^{-1})	237.7
Appearance	Bluish colored gas

Figure 14. Lewis resonant representation of the molecule of ozone (Langlais et al. 1991).

Commercially, ozone has been used for decades as a reagent in synthesis, for the purification of drinking water, as disinfectant, in wastewater treatment and as a bleaching agent of natural fibers (VV.AA. 1993).

Ozonation is a water treatment technique used since the early twentieth century (Gottschalk et al. 2000), although in the beginning its main objective was disinfection it evolved as a treatment to remove contaminants. This process involves the injection of a more or less concentrated stream of O_3 inside the reaction media. This substance acts degrading organic matter (from pollutants to microorganisms' structures) through two routes. On one hand, O_3 reduction potential is extremely high and acts as a selective oxidant against certain types of molecules. On the other hand, in the presence of OH^-, basic pH, O_3 undergoes decomposition that generates secondary oxidants, like hydroxyl radicals which are even more oxidant species.

In practice, both direct and indirect oxidation reactions will take place at the same time. One kind of reaction will dominate over the other, depending on various factors such as temperature, pH and chemical composition of the water. For example, acidic conditions promote the ozone to react directly with certain functional groups from organic compounds through selective reactions of nucleophile, electrophile or dipolar addition. Meanwhile, in basic medium predominates the decomposition of ozone intro hydroxyl radicals even more oxidant species that react unselectively with organic matter. It is also referred in literature that in real waters with a high organic matter content (≥ 3 $mg \cdot L^{-1}$), O_3 decomposition is mainly controlled by radical-type chain reactions (Acero and Von Gunten 2001).

O_3 MOLECULES DIRECT REACTION

The potential of ozone to act as a nucleophile, electrophile or dipole is due to its resonant structure and its ability to dislocate electrons depicted in fig. 13. Generally, organic compounds degradation takes place over unsaturated groups such as aromatic rings, alkenes, alkines, etc. (Langlais et al. 1991) and over aliphatic compounds with specific functional groups (i.e. amines and sulfides, (von Gunten 2003)). Fig. 15 summarizes those structures and species susceptible of being attacked by molecular ozone. However, kinetic constants from all those direct reactions with O_3 are lower than those that take place through hydroxyl radicals (Hoigné and Bader 1975; Hoigné and Bader 1983; Hoigné et al. 1985; Yao and Haag 1991).

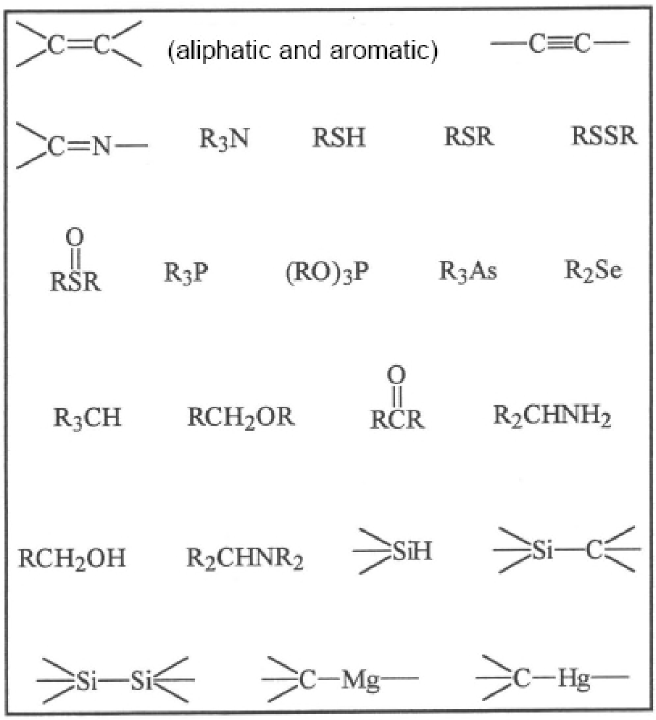


Figure 15. Species and functional groups susceptibles to react with molecular ozone (Rice 1997).

Dipole attack: cycle-addition, Criegee mechanism.

Because of its resonant dipole configuration, ozone can perform additions 1-3 to unsaturated compounds, the double bond breaks and results in carbon-oxygen cycles called "primary ozonide", item (I) in fig. 16.

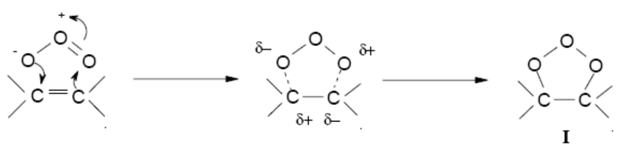


Figure 16. Dipolar cylce-addition to unsaturated bonds. 1st stage of Criegee mechanism

In a polar solvent, such as water, primary ozonide decomposes into a molecule substituted with a carbonyl group (aldehyde or ketone) and a zwitterion (II in fig. 17), an electrically neutral compound that has formal positive and negative charges in their atoms, allowing polar behavior. Then, this species decomposes resulting in a hydroxy-hydroperoxide (III in fig. 17) that ultimately results in the formation of a carbonium compound and hydrogen peroxide.

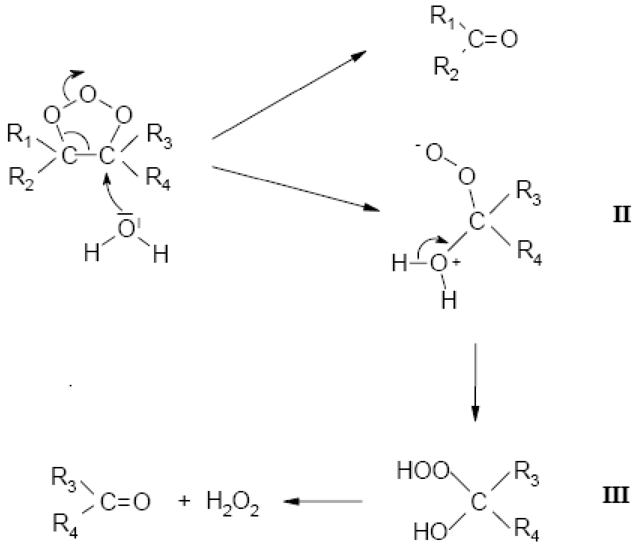


Figure 17. 2^{nd} stage of Criegee mechanism

Electrophile attack

Electrophilic reaction with ozone, fig. 18, is located over the atoms of the molecule with a higher charge density. Aromatics with electron-donating substituents are particularly prone to this type of reactions. Carbons located at positions ortho-and para- with respect to groups such as hydroxyle, NH_2^-, etc., have high charge density and are susceptible to ozone attack. However, if the substituents are electron acceptor, reaction can still be carried out in anyway in meta- carbons less inactivated. The initial attack of ozone, cause the formation of byproducts ortho-and para- dihydroxylates which again can be ozonated. By this process, the aromatic end up as quinoids and aliphatic products with carbonyl-type functional groups.

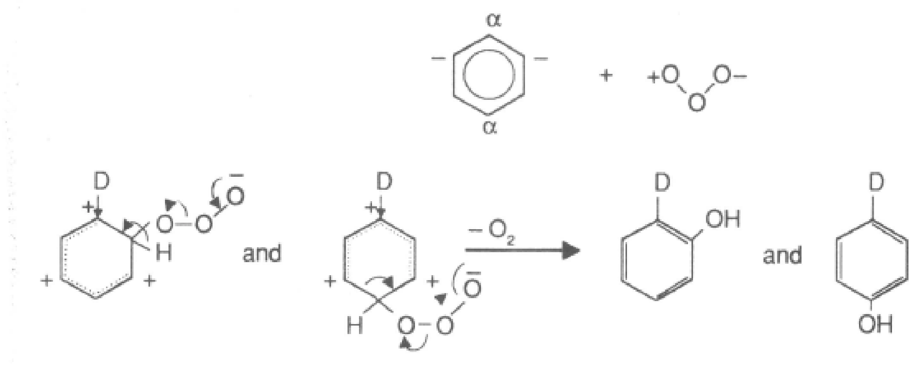


Figure 18. Electrophile reaction of ozone with aromatic compounds (Langlais et al. 1991).

Nucleophile attack

The nucleophile reaction carried out in the charge deficit atoms, especially those associated with electron withdrawing groups, particularly at carbon compounds that contain electron-retreating groups, such as -COOH and $-NO_2$.

O_3 MOLECULES INDIRECT REACTION

Contrary to those based on direct ozone attack, hydroxyl radical reactions are largely unselective. In aqueous medium, OH^- promotes the decomposition of ozone, based on its inherent instability in water (von Gunten 2003), with a subsequent series of chain reactions that lead to the formation of hydroxyl radicals, among other radical species. Reaction mechanism is complex and can be subject to interference from different species in the media and even the concentration of the different compounds in the reaction. However, a simplified pathway is explained as follows.

Initiation

The reaction between ozone and hydroxyl anion, the initiator, generates superoxide ion and the radical hydroperoxide. Several authors proved this reaction to be the limiting step of the whole process (Elliot and McCracken 1989; Hoigné et al. 1985; Sehested et al. 1984).

$O_3 + OH^- \rightarrow O_2 \cdot + HO_2 \cdot$ (k_1=10 $M^{-1} \cdot s^{-1}$) r. 20

Hydroperoxide radical has an acid/ base equilibrium of pKa = 4,8 described in reaction 21.

$HO_2 \cdot \rightleftharpoons O_2{}^- \cdot + H^+$ r. 21

This equilibrium starts the radical chain reaction (Staehelin and Hoigné 1985).

Propagation

$O_3 + O_2^{-} \cdot \rightarrow O_3^{-} \cdot + O_2$ (k_2=1.9·10^9 M^{-1}·s^{-1}) r. 22

From this point $O_3^{-} \cdot$ decomposes to different species depending on the pH of the media (von Gunten 2003):

- For pH≤ 8:

$O_3^{-} \cdot + H^{+} \rightleftharpoons HO_3 \cdot$ (k_{3+}=5·10^{10} M^{-1}·s^{-1}; k_{3-}=3.3·10^2 s^{-1}) r. 23

$HO_3 \cdot \rightarrow OH \cdot + O_2$ (k_4=1.4·10^5 s^{-1}) r. 24

- For pH≥ 8:

$O_3^{-} \cdot \rightleftharpoons O^{-} \cdot + O_2$ (k_{5+}=2.1·10^3 s^{-1}; k_{3-}=3.3·10^9 s^{-1}) r. 25

$O^{-} \cdot + H_2O \rightarrow OH \cdot + OH^{-}$ (k_6=10^8-2·10^9 M^{-1}·s^{-1}) r. 26

No matter the pH it can be seen $O_3^{-} \cdot$ ends up in the generation of hydroxyl radicals that will contribute to the oxidation of the organic matter. Furthermore, ozone itself is susceptible to react with hydroxyl radicals leading to the formation of hydroperoxide radical that starts the cycle all over again.

$O_3 + OH \cdot \rightarrow OH_2 \cdot + O_2$ r. 27

This chain-reaction develops can be maintained even by organic substances that can act as promoters. When a promoter reacts with OH·, it transforms it into hydroperoxide radicals, such as in reaction 28, that again start the cycle all over again. At the same time, the promoter is oxidized and degraded.

$H_2R + OH \cdot + O_2 \rightarrow R + HO_2 \cdot + H_2O$ r. 28

Termination

The completion of the chain reaction is reached when the radicals react with intermediates or no promoting organic substances. In those cases reaction does not lead to formation of hydroperoxide species, as it happens in the following reactions with alkalinity content of the solution:

$OH \cdot + CO_3^{2-} \rightarrow OH^{-} + CO_3^{-}$ r. 29

$OH \cdot + HCO_3^{2-} \rightarrow OH^{-} + HCO_3^{-}$ r. 30

Or when radical species react between themselves

$OH \cdot + OH_2 \cdot \rightarrow O_2 + H_2O$ r. 31

OZONATION APPLIED TO PESTICIDE POLLUTED EFFLUENTS, STATE OF THE ART

The advantages of using ozone as an oxidative technique mainly rely in its oxidative potential and the harmlessness of the byproducts of its own decomposition (oxygen and water on its ultimate extent), makes this technique very interesting for the degradation of organic pollutants, pesticides among them (Acero and Von Gunten 2001; Maldonado et al. 2006).

However, few are the real applications of ozone for the treatment of real wastewaters, because the removal of high concentrations of pollutants always implies the use of large amounts of ozone, making difficult the economical implementation of the technique (Maldonado et al. 2006; Maldonado et al. 2007). Furthermore, in some cases ozonation byproducts can be even more dangerous than the parent compounds (Gottschalk et al. 2000). For instance oxidation products of organophosphorous pesticides (diazinon, methyl-parathion, parathion, chlorpyris) include oxons, picric acid, phosphoric acid and nitrophenol. From all of them, especially oxon forms are more potent acetylcholinesterase inhibitors than the pesticide themselves (Wu et al. 2009; Wu et al. 2009).

For these two reasons, the cost of an intensive treatment, and the possibility of more toxic byproducts, several works are based on the combination of ozonation with other AOPs in order to ensure the adequate extent of the oxidation with the required warranties, such in the case of photocatalysis (Agustina et al. 2005; Farré et al. 2007a). Other authors propose the enhancement of the ozonation process combining it with reagents typical from other AOPs. (Acero et al. 2008) compared ozonation performance with the performance of this process improved with UV and H_2O_2. (Amir Tahmasseb et al. 2002; Chelme-Ayala et al. 2011) also essayed the combination with H_2O_2 for the degradation of phenylurea pesticides, bromoxyl and fribluralin, respectively. (Qiang et al. 2013) introduced the novelty of supported Fe(II) as a catalyzer, to increase the formation of active radicals.

Finally, heterogeneous ozonation is one of the main trends that are being exploited in the last 5 years. It consists in the use of ozonation over pesticides not dissolved in water; they could be suspended in aqueous media like (Meng et al. 2010), adsorbed in a solid phase, as in the case of (Pflieger et al. 2011); or in the surface of vegetables, as Chen et colleagues studied (Chen et al. 2013; Chen et al. 2013).

Table 13 comprises the referred papers and their main features.

Table 13. Ozonation applied to pesticide polluted effluents. State of the art.

Reference	**Pesticides**	**Methods**
(Acero and Von Gunten 2001)	Atrazine	Lab scale O_3
(Acero et al. 2008)	Chlorfenvinphos	Lab scale O_3 UV O_3/H_2O_2 UV/H_2O_2
(Amir Tahmasseb et al. 2002)	N- dimethylphenylureas N-methyl-methoxyphenylureas	Lab scale O_3 O_3/H_2O_2
(Chelme-Ayala et al. 2011)	Bromoxyl Fribluralin	Lab scale O_3 O_3/H_2O_2
(Chen et al. 2013)	Permethrin Chlorfenvinphos Chlorothalonil	Domestic pilot plant scale O_3 (gas media)
(Farré et al. 2007a)	Pentachlorophenol Isoproturon Diuron Alachlor Atrazine	Lab scale Photocatalytic ozonation
(Maldonado et al. 2006)	Alachlor Atrazine Chlorfenvinphos Diuron Isoproturon	Pilot plant O_3
(Meng et al. 2010)	Malathion Chlorfenvinphos	Lab scale Heterogeneous ozonation (suspended pesticides)
(Pflieger et al. 2011)	Trifluralin	Lab scale Heterogeneous ozonation (pesticides adsorbed on mineral particles)
(Qiang et al. 2013)	Omethoate	Lab scale Photocatalyzed ozonation (Fe^{2+} loaded on activated carbon)
(Wu et al. 2009)	Methyl parathion Parathion Diazinon	Lab scale O_3

1.6. BIOLOGICAL TREATMENT OF WASTEWATER

Biological treatment of wastewater, groundwater, and aqueous hazardous wastes is often the most economical alternative when compared with other treatment options. In these treatments, the metabolic activity of living organisms is able to degrade to a certain extent the organic load of an effluent (European Commission, Joint Research Centre 2003).

In wastewaters, sewage, and most of bioreactors, bacteria coexist with algae, fungi, protozoa, etc., even with multicellular microorganisms. Although all this microflora and microfauna takes part on the ecosystem of the biological reactor (Watanabe 2001), bacteria play the most important role in the assimilation of organic matter content and the metabolizing of pollutants (Bankar et al. 2009; Deng et al. 2010). One of the main reasons that make bacteria so active in these environments is their intimate relationship with the media due to their small sizes, and their high surface area-to-volume ratio (Imfeld and Vuilleumier 2012). While aerobic bacteria require oxygen for life support, anaerobes can sustain life without oxygen, and facultative bacteria are able to live either in the presence or in the absent of oxygen. This broad adaptability could be considered also another reason for the critical role bacteria play in bioreactors.

The ability of a certain compound to undergo biological degradation in a concrete bioreactor is dependent on a variety of factors, such as concentration, chemical structure and substituents of the target compound. The pH or the presence of inhibitory compounds can also affect the biological degradation, together with the distribution of the main consortium of microorganisms. Depending on the availability of O_2 in the reacting media, there are five typical sets of working conditions that determine the performance of the processes: aerobic, anoxic, anaerobic, a combination of this three in the same reactor but at different times, and pond processes (lagoon processes, in which the first three conditions are taking place simultaneously in different zones of the lagoon)

Regarding to aerobic reactors, which performance requires the presence of oxygen, the most common working conditions is what is called *suspended-growth*. It is based on the growth and retention of a suspension of microorganisms which are able to convert organic matter and other constituents of the effluent into energy and/or cell tissues, with the release of CO_2 and CH_4 (Shieh and Keenan 1986). In wastewater treatment this distribution is typically referred to as *activated sludge*, and presents a variety of reactor configurations and flow patterns as listed in table 14.

Table 14. Common configurations of activated sludge process (Redda 2008)

Activated sludge processes
• Conventional
• Contact stabilization
• Complete mix

- Extended aeration
- Sequencing Batch reactor

Precisely, sequencing Batch Reactors, described in Chapter 3 in a lab scale version, are based in these suspended biomass distribution. As described in (Burton and Tchobanoglous 1991), in this processes, organic waste is introduced into a reactor where an aerobic bacterial culture is maintained in suspension by the use of diffused aeration or mechanical stirring. The reactor content is known as mixed liquor and the association of the different bacterial species is called a consortium. After a specific period of time, the mixed liquor is lead to settle (in an adjacent tank or in the same device without stirring) and the sludge can be separated from the treated wastewater. The capacity of the adaptive consortium for the removal of pollutants can be enhance by subjecting reactors to sequential or alternating redox (aerobic-anaerobic) environment (Hu et al. 2005).

In contrast to activated sludge processes where the waste-consuming bacteria grow in suspension, devices such as the active bacteria consortium in attached growth processes cling to a certain surface, natural or manmade, to perform the degradation of the organic matter that the surrounding medium contents. This kind of growth works in aerobic or anaerobic conditions depending on the settings of the bioreactor, usually combining both. The microorganisms grow on filter media (gravel, sand, peat, or specially woven fabric or plastic) and metabolize dissolved organic material for their catabolism, their self-structure or external biofilm that connect all the attached microbiota. There are two basic designs of attached growth or fixed film systems: those that hold the media in place, allowing the effluent to flow over the bed (so is the case of slow sand filtration column used in this thesis), or those where the media is in motion in relation to the wastewater (e.g., rotating biological disks). It is well known that the biofilm configured systems are well suited for the treatment of wastewater containing poorly degradable compounds (Bluwer 1989; Makinen et al. 1993). Immobilization of microorganisms as biofilm results in high biomass hold up, which enables the process to be operated at significantly higher liquid throughputs and Organic Loading Rates (OLR) (Shieh and Keenan 1986). Attached biofilm acts as buffer to reduce the concentration of toxic chemicals during process operation thereby providing advantage for the treatment of low biodegradable industrial wastewater containing recalcitrant compounds (Bishop 1997; Chaudhry and Beg 1998). Biofilm systems are generally less energy intensive and more resistant to shock loads to which wastewater treatment systems are frequently subjected (Chaudhry and Beg 1998). Supported biomass systems are particularly useful where high hydraulic loading variations occur and where slowly growing organisms with special metabolic capacities are to be protected from washout (Wilderer and Roske,I.,Ueberschar,L.D. 1993).

1.6.1. SLOW SAND FILTRATION

Slow Sand Filtration (SSF) is one of the earliest forms of water treatment, specially conceived for potable water, although it has applications also on wastewater treatments. SSF has been widely used in Europe sin the early 1800s, and still remains an important process for water purification throughout the world (Campos et al. 2002). Simplicity and low capital and operating costs (Campos et al. 2002), together with the robustness against fluctuations in feed quality (Moncayo-Lasso et al. 2008), are the main advantages of this technology compared to more sophisticated methods of water treatment. The easiness of its design and its minimal power, chemical and control requirements bears on low installation and operating costs (Campos et al. 2002). In fact, manpower is one of the main contributions to the operating costs, especially in the case of high concentration of suspended solids. Efficient control and automation noticeable reduce this output, reducing it to cleaning tasks.

In a SSF, raw water flows by gravity (100-200 lmh) through a column of sand with high surface area, which is susceptible to be colonized by microorganisms (attached growth). A combination of two factors contributes to the efficiency of SSF. On one hand, there is the effect of the physical filtration and absorption, which is determined by the effective size of the filling. To some extent, inert particles and molecules can be removed by collision and attachment mechanisms that occur within the filtering media (Logsdon et al. 2002). On the other hand, a biological factor also contributes to SSF performance. It consists on the activity of the biomass layer that grows on the sand bed, thanks to an initial inoculum or to the characteristics of the water loads. This biomass layer is typically referred as *schmutzdecke* (Huisman and Wood 1974), it grows specially in the interface water/filtering media, and its concentration decreases with increasing bed depth (Hallé et al. 2009).

It could be said that high efficiency of SSF as water treatment primarily relies on the fine effective size of the sand (0.1-0.3 mm) (Campos et al. 2002), together with the length of the operational run time (flow rates between 0.1-0.3 mh^{-1}) (Ellis 1987). Other researches focused in drinking water suggested that temperature, source water features and surface ripening extent have also significant effect on the surface ripening extent have likewise significant effect on the performance of the filter (Amy et al. 2006). (Leverenz et al. 2009) include as crucial factors bioaugmentation[3], media depth, mineral composition of the media, pretreatment, if any, hydraulic and organic loading rates and dosing techniques.

Be that as may, the synergy of both physical and biological phenomena have stated this technology as a suitable method for the removal of suspended solids (Ellis 1987; Logsdon et al. 2002), pathogens (Bauer et al. 2011; Martínez et al. 2010) and dissolved organic matter (Linlin et al. 2011; Zheng et al. 2009; Zheng et al. 2010). In fact, in recent years, a resurgence of interest in this technique has occurred based on its proven

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[3] Addition of necessary nutrients not present in the effluent to treat

efficiency removing dissolved organic matter remaining after an oxidation pretreatment (Graham 1999; Moncayo-Lasso et al. 2012; Moncayo-Lasso et al. 2008).

1.6.2. COUPLING AOPS/BIOLOGICAL TREATMENT

Though they are usually and economical option for water treatment, biological processes do not always provide satisfactory results, since many organic substances produced by the chemical industry are toxic or resistant to the biological treatment (Lapertot et al. 2006; Sarria et al. 2002).

On the other hand complete oxidation by chemical processes is generally expensive due to the fact that the oxidation intermediates formed during treatment tend to be more and more resistant to their complete degradation, thus requiring an increasing consumption of energy (radiation, etc.) and chemical reagents (catalysts and oxidizers) (Muñoz et al. 2005; Oller et al. 2011).

As a solution to these two issues, one attractive alternative would be to integrate both strategies. Chemical oxidation processes could be applied as a pre-treatment to convert the initially persistent organic compounds into more biodegradable intermediates, which would then be treated in a biological oxidation process with a considerably lower cost (Sarria et al. 2002). The percentage of mineralization should be minimal during the pre-treatment stage in order to avoid unnecessary expenditure of chemical and energy, thereby lowering the operating cost indeed. However if the pre-treatment time is too short, the reaction intermediates generated could still be structurally very similar to the original non-biodegradable and/or toxic components.

The coupling requires application of toxicological and biological methods to evaluate effluent toxicity and biodegradability before it can be transferred to a biological treatment for its complete oxidation. In some cases, it has been found that toxicity of the original effluent grows during early pre-treatment up to a maximum due to the formation of toxic intermediates. Toxicity analyses during AOP treatment of wastewater provide valuable information on samples in which the percentage of inhibition has changed enough to make performing biodegradability tests worthwhile, because a significant change in toxicity would usually be related to a substantial change in biodegradability (Oller et al. 2011).

Scott and Ollis, (Scott and Ollis 1995), identified four types of wastewater as potentially treatable by combined AOPs/biological degradation:

- Water containing biorecalcitrant compounds usually characterized by its large size (i.e. soluble polymers) and lack of active centers
- Highly biodegradable effluent which still requires chemical post-treatment as it contains a large amount of biodegradable organic matter in addition to small concentrations of recalcitrant compounds.

- Water containing inhibiting compounds which are somewhat toxic to a certain percentage of some biological cultures.
- Effluent containing inert intermediates which must be effectively degraded to avoid its accumulation n the media and the inhibition of microorganism growth.

Selection of the best treatment option for remediation of a specific industrial wastewater is a highly complex task. The choice of one or more processes to be combined in a certain situation depends on the quality standards to be met and the most effective treatment with the lowest reasonable cost. Therefore, according to (Oller et al. 2011), the main factors which must be considered in the decision on the wastewater treatment technologies are:

- The quality of the original wastewater
- Removal of parent contaminants
- Conventional treatment options
- Treatment flexibility
- The facility decontamination capacity
- Final wastewater treatment system efficiency
- Economic studies
- Life cycle assessment to determine environmental compatibility of the wastewater treatment technology
- Potential of use of treated water

Advanced Oxidation Processes can be a good alternative for the chemical pre-treatment. These techniques are widely recognized as highly efficient treatments for biorecalcitrant polluted water. The interest in this combined AOPs/biological technologies has grown lately due to the real possibility of reusing industrial wastewater as a safe water resource under adequate sanitary conditions (Oller et al. 2011). The success of combining an AOP with biological treatment to treat substances such as pharmaceuticals, pesticides and dyes is well documented (Elmolla and Chaudhuri 2011; Liberatore et al. 2012; Oller et al. 2011). Sequencing batch reactors (SBRs) have been extensively studied as the biological treatment in coupled processes (Ballesteros Martín et al. 2009b; Ballesteros Martín et al. 2010; Elmolla and Chaudhuri 2011). Other biological treatments, like immobilized biological reactors (IBRs) (Wang et al. 2008), fixed bed reactors (FBRs) (Chen et al. 2009) or membrane bioreactors (MBRs) have also been studied (Laera et al. 2011). Even an intimate coupling of photocatalysis and biodegradation in a photocatalytic circulating-bed biofilm reactor has centered some studies (Parra et al. 2000).

1.6.3. COMBINED AOP/BIOLOGICAL REACTOR FOR WATER CONTAINING PESTICIDES, STATE OF THE ART

Pesticides and herbicides, among the priority substances, are considered a serious threat to surface and groundwater, since their high solubility makes their propagation in the environment extremely easy (Pesticide Action Network 2010). Several monitoring programs around the world have shown the presence of at least traces of pesticides in surface and groundwater masses. This diffuse contamination is very important source of environmental harm, however, also highly concentrated effluents, even reaching 500 $mg \cdot L^{-1}$ (Oller et al. 2011), can compromise the environment for an irresponsible wastewater disposal from pesticide producing factories or even from direct irrigation water used in agriculture. This doctoral thesis is focused in this kind of effluents.

Numerous authors proposed the feasibility of coupling advanced oxidation processes with biological reactors for the treatment of biorecalcitrant substances. In the case of pesticide polluted effluents intense work have been also done, using different biomass distributions and diverse oxidation techniques.

(Lapertot et al. 2007) combined photo-Fenton reaction with packed bed bioreactors for the treatment of a mixture of pesticides. This coupling allows shortening the chemical reaction time; this aspect has a critical impact on the economic feasibility of the whole integrated process. The obtained concomitant decrease of toxicity and increase of biodegradability of the partially photo-treated solution of mixed pesticides allowed a global 80% decrease of the total DOC by the coupled system (50% of the total carbon conversion corresponds completely to the bioreactor). Several works included immobilized biomass reactors coupled with photo-assisted AOPs, like in (Oller et al. 2007). This work compared TiO_2 photocatalysis with photo-Fenton reaction, which integrated process with a semi continuous IBR resulted as more efficient for pesticide degradation and DOC mineralization. Like (Malato et al. 2007), (Zapata et al. 2010) focused the research on the industrial scale application of a coupled system with solar photo-Fenton combined with immobilized biomass reactor. It showed that photo-Fenton could reduce toxicity of the effluent from 96 to 50% inhibition and increase biodegradability from 50% to 95%. However as total depletion of active ingredients was requested, the combination of both processes was not successful.

(Parra et al. 2002) also proposed a IBR, this time integrated with heterogeneous photocatalysis. In this case 100% of isoproturon (target pesticide) was depleted, together with a final mineralization of 95% DOC. In a previous work (Parra et al. 2000), this very author compared the photochemical system with the coupled system (fixed bed reactor) for two pesticides; isoproturon effluent, as in 2002 reference, appeared to be highly compatible with the biological reactor after photo-Fenton reaction, in opposition to this, metobromuron solution after photo-Fenton is not appropriate for a biological system. This work studies several photochemical systems and the obtained results state that however the strategy of coupling photochemical and biological systems is not necessarily the universal solution.

More recently, (Moreira et al. 2012) also essayed different AOPs coupled with immobilized biomass reactor. In this case, the biodegradability of the starting effluent imposed to place the biological process prior to AOP. Considering all the advanced oxidation techniques, photo-Fenton seemed to achieve better results in terms of pesticide degradation and mineralization of a mixture of 19 different pesticides. A rearrangement of the processes also defines this other work, (Vilar et al. 2012), which proposes a 3-step system: bioreactor + solar photo-Fenton + bioreactor. The total process was able to achieve pesticide abatement below respective limits and a global mineralization of the 79%.

A coupled system with a membrane biofilm reactor is proposed by (Sánchez Pérez et al. 2013) to treat industrial ecotoxic wastewater (a mixture of five commercial pesticides). Among other conclusions, this study, specially focused on the economical evaluation stated that the increase of pollution load does not always involve an increase in photo-Fenton costs because they also depend on organic matter mineralization. It also showed that the use of an MBR permits lower photochemical oxidation requirements than other biological treatments 20% photo-Fenton cost reduction for highly polluted wastewater. Then pollution loaded decreases, the contribution of reactant consumption to the photo-Fenton processes increase with regard to amortization costs. 30% total cost reduction can be gained treating higher daily volumes, obtaining competitive costs that vary from 1-1.9€/m^3, depending on the pollution load.

By now, all the referred articles work on the coupling of photo assisted AOP techniques, however, there are also examples of ozonation combined with biological reactor, such in the case of (Mezzanotte et al. 2005). The ozonation is integrated in this paper with a submerged biofilter. They treat an industrial effluent containing high pesticide concentrations. In the case of (Lafi and Al-Qodah 2006), ozonation and UV enhanced ozonation is combined with sequence batch reactors.

(Marsolek et al. 2008) proposed an interesting point of view, suggesting the integration of the advanced oxidation process and biological degradation taking place in the same reactor. A photocatalytic circulating-bed biofilm reactor, PCBBR, with macroporous carriers allows the intimate coupling of the two techniques by the protection that the porous structure offers to biofilm from toxic reactants, radicals, intermediates and UV-light. Without the presence of the colonized carriers, photocatalysis removed target compound (2, 4, 5-trichlorophenol, TCP) and COD to 32% and 26% of the influent levels, but the addition of biofilm carriers decreased residuals to 2% and 4% respectively.

Several works included a series of tests to check biodegradability of the effluents before and after chemical treatment. (Oller et al. 2011) considers *virbrio fischeri* toxicity essays and *Zhan Wellens*[4] test as useful techniques to select the moment when photo-

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[4] The “Zahn-Wellens test” is a classical test to determine the inherent (in principle possible) degradability of water-soluble substances. The test effluents are incubated with certain mineral salts with relatively high amounts of activated sludge (0.2 to 1.0 g/l) as inoculum during 28 days as a batch test. The test concentration varies between 50 and 400 mg/l DOC or 100 and 1000 mg/l COD, respectively. Samples are taken in regular intervals and the decrease of DOC or COD in the filtrated samples is measured over time. The portion adsorbed to the sludge is estimated from a sample taken after 3 hours. It will be also used as a reference value for the calculation of the biological degradability. At the same time, the blank value of the activated sludge, the degradability of a reference substance and the abiotic elimination of the

Fenton pretreatment has sufficiently enhanced the biocompatibility of the pesticide mixture for a subsequent combination with the biological treatment. (Parra et al. 2002; Vilar et al. 2012) include also BOD_5/COD ratio as another way to evaluate the extent of the chemical treatment requested for assuring the biocompatibility with the biological processes.

A summary of the references discussed above and others, along with their most remarkable ideas, is shown at the table 15.

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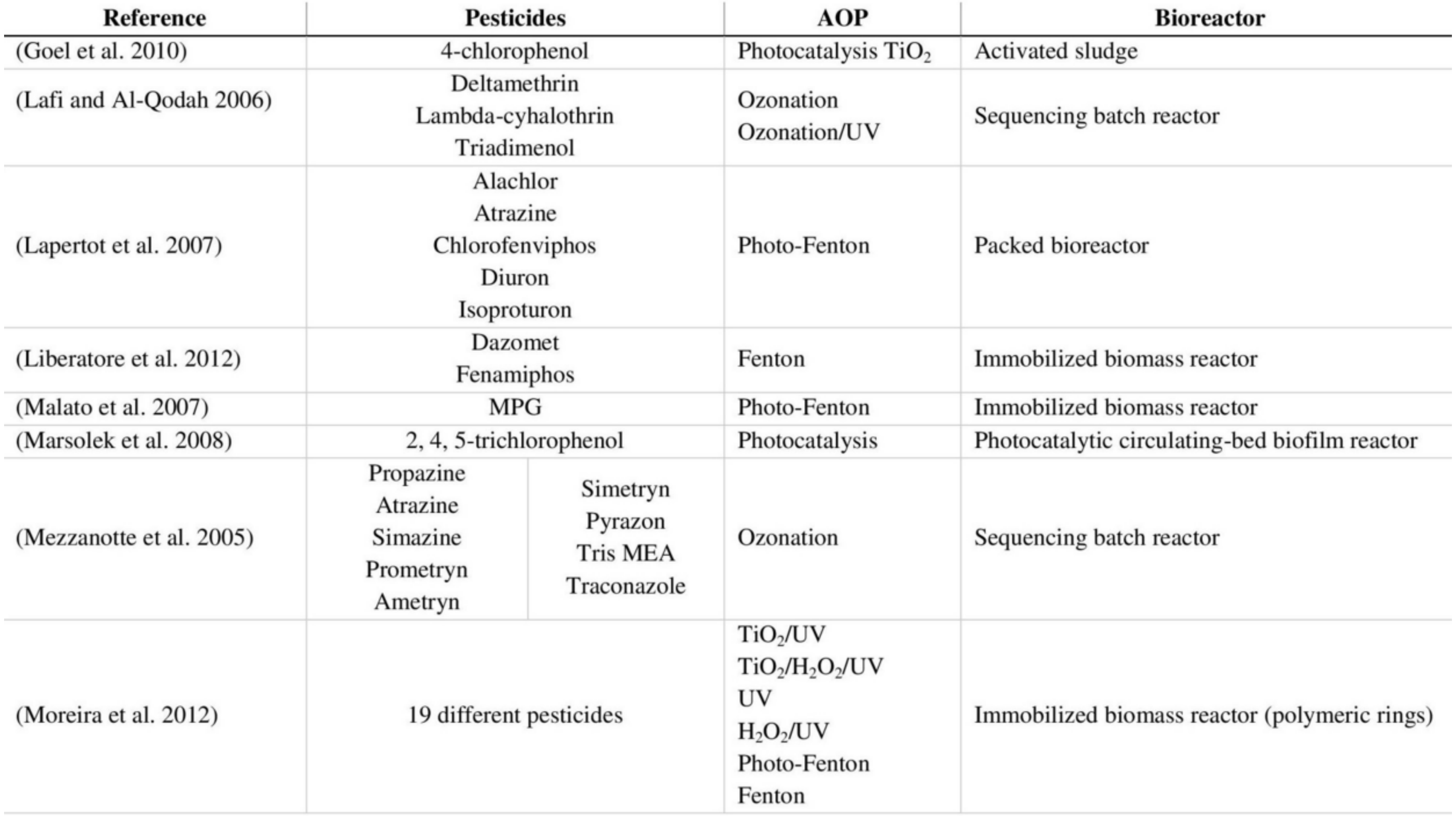
test substance (intoxicated sample without activated sludge) are each determined in at least one additional test vessel(European Commission 2008).

Table 15. AOP combined with biological reactor for the treatment of effluents polluted with pesticides. State of the art.



Reference	Pesticides	AOP	Bioreactor
(Goel et al. 2010)	4-chlorophenol	Photocatalysis TiO_2	Activated sludge
(Lafi and Al-Qodah 2006)	Deltamethrin Lambda-cyhalothrin Triadimenol	Ozonation Ozonation/UV	Sequencing batch reactor
(Lapertot et al. 2007)	Alachlor Atrazine Chlorofenviphos Diuron Isoproturon	Photo-Fenton	Packed bioreactor
(Liberatore et al. 2012)	Dazomet Fenamiphos	Fenton	Immobilized biomass reactor
(Malato et al. 2007)	MPG	Photo-Fenton	Immobilized biomass reactor
(Marsolek et al. 2008)	2, 4, 5-trichlorophenol	Photocatalysis	Photocatalytic circulating-bed biofilm reactor
(Mezzanotte et al. 2005)	Propazine Atrazine Simazine Prometryn Ametryn Simetryn Pyrazon Tris MEA Traconazole	Ozonation	Sequencing batch reactor
(Moreira et al. 2012)	19 different pesticides	TiO_2/UV TiO_2/H_2O_2/UV UV H_2O_2/UV Photo-Fenton Fenton	Immobilized biomass reactor (polymeric rings)

Reference	Pesticides	AOP	Bioreactor
(Oller et al. 2007)	Cymoxanil Methomyl Oxamyl Dimethoate Pyrimethanil	Solar photocatalysis Solar photo-Fenton	Immobilized biomass reactor
(Parra et al. 2000)	Metobromuron Isoproturon	UV/TiO_2 UV Fe^{3+}/H_2O_2 $UV/Fe^{3+}/H_2O_2$	Immobilized biomass reactor
(Parra et al. 2002)	Isoproturon	Photocatalysis TiO2	Biolite ® attached bacteria reactor
(Sánchez Pérez et al. 2013)	Oxamyl Methomyl Imidacloprid Dimethoate Pyrimethanil	Photo-Fenton	Membrane biorreactor
(Vilar et al. 2012)	Benthazone S-methachlor Terbutylazine Atrazine Desisopropilatrazina Alachlor Pririmetanil Imidacloprid Isoproturon Tebuconazole Chlorotoluron Diuron Simazine Dimethoate Linuron Terbuthylazone-desethyl	Solar photo-Fenton	Immobilized biomass reactor
(Zapata et al. 2010)	Oxamyl Dimethoate Imidacloprid Pyrimethanil	Scale-up Solar photo-Fenton	Immobilized biomass reactor

1.7. MOLECULAR BIOLOGY TECHNIQUES

The history of molecular biology begins in the 1930s with the convergence of various, previously distinct, biological disciplines: biochemistry, genetics, microbiology, and virology. It could be said that this set of biological techniques deal with the molecular basis of biological activity; DNA, RNA and proteins biosynthesis (ASTBURY 1961). With the hope of understanding life at its most fundamental level, numerous physicists and chemists also took an interest in what would become molecular biology, which nowadays comprises tools such as expression cloning, polymerase chain reaction (PCR) based techniques, gel electrophoresis base techniques, macromolecules blotting and probing, etc.

While molecular biology discipline was established at the beginning of the 1930s, the term was coined by Warren Weaver in 1938. Weaver, director of Natural Sciences for the Rockefeller Foundation at the time, believed that biology was about to undergo a period of significant change and promoted a noticeable founding incentive for research in this field, mainly to California Institute of Technology, Caltech. From that time on the interest in this field spread to other institutions, which allowed that between the late 1950s and early 1960s, molecular biologists established the principles to characterize, isolate, and manipulate the molecular components of cells and organisms (Kay 1996). These components included DNA, the repository of genetic information; RNA, a close relative of DNA whose functions range from serving as a temporary operational DNA copy to act structurally and as enzyme, as well as a functional and structural part of the translational apparatus; and proteins, the major structural and enzymatic type biomolecule.

The chief discoveries of molecular biology (relationship between genes and proteins, double helical structure of DNA molecule, messenger RNA as an intermediate between DNA and proteins, etc.) took place in a period of only twenty-five years. Another fifteen years were required before new and more sophisticated technologies, united today under the name of genetic engineering, would permit the isolation and characterization of genes, in particular those of highly complex organisms. Table 16 summarizes the most important milestones in genomics that also were critical for molecular biology from XIX century to the beginning of XXI (Hales 2009; Lane 1994).

Nowadays, molecular biology techniques (MBTs) have tremendous value not only in the investigation of basic scientific questions, but also in application to a wide variety of problems affecting different human aspects. An understanding of microbial dynamics and their interactions is indispensable in bioremediation techniques, energy generation processes, and in biotechnological industries such as pharmaceutical, food, chemical and even mining (Rastogi and Sani 2011). Disease prevention and treatment, generation of new protein products, and manipulation of plants and animals for desired phenotypic traits are all applications that are routinely addressed by the application of molecular biology methods. Because of the wide applicability of these methods, they are rapidly becoming a pervasive aspect of our technologically based society.

Table 16.Molecular biology millestones.

Year	Scientist(s)	Discovery
1858	Charles Darwin Alfred Russel Wallace	Joint announcement of the theory of natural selection: members of a population who are better adapted to the environment survive and pass on their traits.
1859	Charles Darwin	Published “The Origin of Species”.
1866	Gregor Mendel	Published the results of his investigations of the inheritance of "factors" in pea plants.
1900	Carl Correns Hugo de Vries Erich von Tschermak	Mendel's principles were independently discovered and verified, marking the beginning of modern genetics.
1910	Thomas Hunt Morgan	Proposed a theory of sex-linked inheritance related to *Drosophila*[5]. This fact was followed by the **gene theory**, including the principle of linkage.
1927	Hermann J. Muller	Used x-rays to cause artificial gene mutations in Drosophila.
1928	Frederick Griffith	Performed what was called Griffith’s experiment. It suggested that that bacteria are capable of transferring genetic information through a process known as **transformation**
1944	Oswald Avery Colin MacLeod Maclyn McCarty	Reported that they had purified the transforming principle in Griffith's experiment and that it was **DNA**.
1950	Erwin Chargaff	Discovered a one-to-one ratio of adenine to thymine and guanine to cytosine in DNA samples from a variety of organisms.
1951	Rosalind Franklin	Obtained sharp X-ray diffraction photographs of DNA.
1952	Martha Chase Alfred Hershey	Used phages in which the protein was labeled with 35S and the DNA with 32P for the final proof that DNA is the molecule of heredity.
1953	Francis Crick James Watson	Solved the three-dimensional structure of the DNA molecule.
1958	Matthew Meselson Frank Stahl	Used isotopes of nitrogen to prove the semiconservative replication of DNA.
1958	Arthur Kornberg	Purified DNA **polymerase I** from E. coli, the first enzyme that made DNA in a test tube.
1970	Hamilton Smith Kent Wilcox	Isolated the first restriction enzyme, HindII, which could cut DNA molecules within specific recognition sites.
1972	Paul Berg Herb Boyer	Produced the first recombinant DNA molecules.
1973	Joseph Sambrook	Led the team that refined DNA **electrophoresis** by using agarose gel and staining with ethidium bromide.
1973	Annie Chang Stanley Cohen	Showed that a recombinant DNA molecule can be maintained and replicated in E. coli.
1985	Kary B. Mullis	Published a paper describing the **polymerase chain reaction (PCR)**, the most sensitive assay for DNA yet devised.
1988	Heinz U. Weier Joe W. Gray	Publised a paper entitled “A Programable System to Perform the Polymerase Chain Reaction”. It was the precursor of thermocycler.

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[5] Also known as *fruit fly*. Drosophila in particular, D. melanogaster, has been heavily used in research in genetics and is a common model organism in developmental biology due to be easy to grow and to have short generation time (10-12 days).

Year	Scientist(s)	Discovery
1988		National Center for Biotechnology Information (NCBI) was founded. Data bank for sequences and taxonomic references.
1989	Alec Jeffreys	Coined the term **DNA fingerprinting** and was the first to use DNA polymorphisms in paternity, immigration, and murder cases.
1995		Automated sequencing technology allows genome projects to accelerate.
1996-97	Ian Wilmut	His team took part in the first **cloning of a mammal** (Dolly the sheep).
2001		The sequence of the **human genome** is released, and the "post-genomic era" officially begins.

1.7.1. MBTS VS. CULTIVATION-DEPENDENT TECHNIQUES

Classical analytic methods were already available to characterize microbial community compositions based on amplified lenses since 16th Century, while cellular cultures were widely employed since Pasteur’s time, 19th Century (Debré 2000). However, the visions of the microbial world they offer are inadequate and insufficient to categorize the vast diversity of cells that can be found in almost any type of media. On one hand, the lack of evident morphologies to categorize visually, and small cell sizes do not allow microscopic identification of the majority of naturally occurring bacteria. On the other hand, media used for the cultivation of microbial strains are selective and hence give biased views of the community composition (Rappe and Giovannoni 2003; Schäfer and Muyzer 2001). In fact, >99% of the microorganisms in any environment observed through a microscope are not cultivable by standard culturing techniques. Additionally, organisms retrieved using common culture methods are rarely numerically abundant or functionally significant in the environment from which they are cultured (Hugenholtz 2002).

Circumventing these inconveniences, MBTs have been established as an alternative to these conventional techniques for in-depth characterization of single organisms or environmental microbial communities.

Molecular biology techniques primary source of information are microorganisms biomolecules, such as nucleic acids, lipids, and proteins; substances that can be found wherever life is taking place. Starting from these molecules, MBTs include analyses of whole genomes or selected genes such as 16S and 18S rRNA (ribosomal RNA), for prokaryotes (Bacteria and Archaea) and eukaryotes (Eukarya) characterization, respectively. Broadly MBTs have been classified into two major categories depending on their capability of revealing the microbial diversity structure and function: (1) partial community analysis approaches; (2) whole community analysis approaches, summarized in fig. 19.

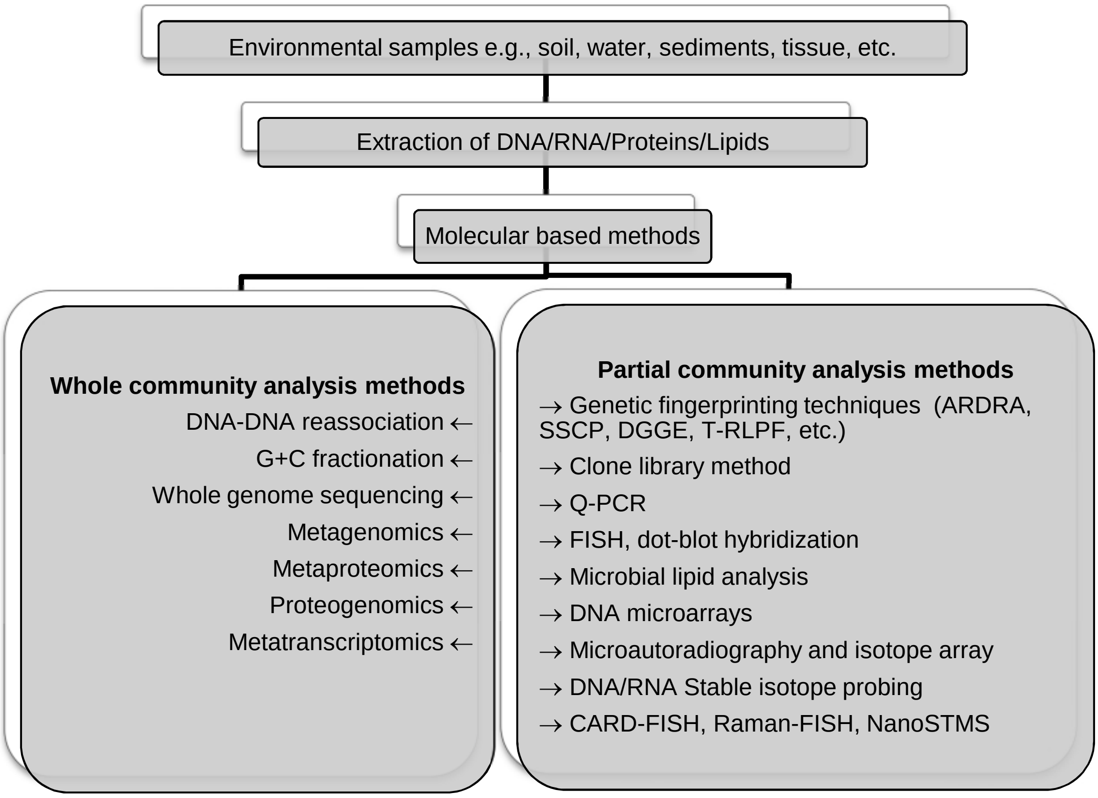


Figure 19: Culture independent molecular toolbox to characterize the structural and functional diversity of microorganisms in the environment. Based on (Rastogi and Sani 2011).

In this thesis, the molecular techniques employed are part of the Partial Community Analysis Approaches group. These generally include polymerase chain reaction (PCR)-based methods where total DNA/RNA extracted from a sample is used as a template for the characterization of microorganisms. In principle, the PCR product thus generated reflects a mixture of microbial gene signatures from all organisms present in a sample.

PCR of conserved genes such as 16 rRNA has been used extensively in microbial ecology primarily because these genes are ubiquitous for prokaryote cells, are structurally and functionally conserved, (common for every kind of bacteria) and contain variable and highly conserved regions (specific of every taxon). In addition, the suitable gene size (~1500 base pairs) and a growing number of 16rRNA sequences available online for comparison in sequence databases allow identifying either the DNA or RNA nucleotide sequences of microbial samples as a phylogenetic classification parameter based on Polymerase Chain Reaction (PCR) (Cardenas et al. 2009; Sibley et al. 2012). By estimating the phylogenetic similarity to known microorganisms based on the homology of 16S rRNA sequences, the closest affiliation of a new isolate or molecular sequence is assigned. This allows the comparison of individuals of the same domain, and differentiation of individuals from the same specie (Sanz and Köchling 2007). To establish this contrast, PCR products amplified from sample DNA are analyzed primarly by (1) clone library method, (2) genetic fingerprinting, (3) DNA microarrays, or by a combination of these techniques.

1.7.2. PRACTICAL APPLICATIONS OF MBTS. MBTS IN ENVIRONMENTAL SCIENCES

The relevance of MBTs is reflected in the 341,789 and 125,494 articles found respectively in sciencedirect.com and scopus.com searching for the term “molecular biology” (checked in August 2012, searches for title, abstract and key words). In those articles, besides microbial ecology in different environment, it can be seen how applicable these techniques are to a wide variety of fields. For instance, food science purposes have provided information about microbes, viruses and molds susceptible to grow on staple (Hartmann and Halden 2012; Luque et al. 2013; O'Flaherty and Klaenhammer 2011). There are also biotracing methods for food safety based on this technology (Nicolau et al. 2013). In veterinary sciences, animal diseases and their vaccines have been studied (Hermann et al. 2012), also molecular biology has contributed to studies related to the effects of using antibiotics in livestock feeding (Thames et al. 2012). Besides there is even a well-established PCR-based method for bird sexing (Morinha et al. 2012).

Another field in which MTBs have a tremendous success and value is in human medicine; from oral infections related works (Vianna et al. 2009), to studies related to swine fever, H_1V_1 influenza virus that caused global hysteria in 2009 (Dutra et al. 2010; Leifer et al. 2010; Mak et al. 2011; Poon et al. 2009), acute lung infections (Galle et al. 2012), tuberculosis (Azad et al. 2012), and malaria (Guimarães et al. 2012). There is even a branch of molecular biology technologies devoted to simplify their methods in order to employ them in resource-limited settings (Coloma and Harris 2009; Djikeng et al. 2011). The aspect which is given most promising results in the use of MBTs is cancer research; with works that go from the studies of methylation of DNA and RNA-Seq (Xie et al. 2011) in heart, kidney and liver disorders, to the complete genome sequencing for lung cancer (Daniels et al. 2012).

The contribution of MBTs to environmental sciences has enhanced the knowledge of population diversity in any ecosystem, marine waters (Zehr et al. 2009), tropical forests (Troxler et al. 2012), etc.; even in extreme conditions, from high arctic environments (Varin et al. 2012), to exceptionally acid waters, such in Río Tinto, Spain (Bühring et al. 2012), or deep oceanic ridges (Takai and Nakamura 2011). However, nowadays, practical purposes are leading the trends of using these technologies in environmental science. Regarding to this subject, MTB’s have also several action fronts: wildlife monitoring and population genetic research (Davoli et al.), behavioral biology (Smith et al. 2008), influence of contaminants in reproductive cycles (Trinchella et al. 2010), taxonomic kinship (Gailing et al. 2012), etc. However, in the field of environmental damage prevention and remediation, molecular technologies have suffered a strong impulse in the last 30 years. After realizing the existence of microbes in almost every environment and their potential in different environmental-enhancing aspects (Podar and Reysenbach 2006), practical application of microbes turned into their use as “tinny miners” through bioleaching (Wang et al. 2012), bioremediations and biofuel producers. Molecular techniques are already being employed over microorganisms consortia which are used to produce biofuels in the shape of biodiesels from different substrates, (Abou-Shanab et al. 2011; Adachi et al. 2012; Kesari et al. 2012; Mutanda et al. 2011; Tabatabaei et al. 2011; Xing et al. 2012), or included in fuel cells for hydrogen production (Goud et al. 2012; Hasyim et al. 2011; Li et al. 2011; Mangayil et al.; Venkata Mohan et al. 2010).

In the case of bioremediation microorganisms devoted to the removal of pollutants can be found in every soil or water media (Iwamoto and Nasu 2001), like they do in waste water treatment plants (Hu et al. 2012), or in heavy metal polluted soils (Zhu et al.). Precisely, bioreactors, a kind of ex-situ bioremediation treatment, are the basic process of almost every waste water treatment plant, and are in constant development as adapted population individual reactors for treating recalcitrant organic substances (Burton and Tchobanoglous 1991). The last trends in this technology are highly dependent on MBTs. On one hand they are used in scientific research and predictions, where these constantly evolving tools are used to gain new knowledge. On the other hand, they can also be used as a commercially available diagnostic tool to obtain information about biological processes that are already taking place (Alleman et al. 2005).

New technologic drifts in bioreactors impose the use of engineered systems that tend to select a few functional microbial groups that may be organized in sludge flocs, biofilms or granules, or to design engineered inoculums for a certain purpose (Khondee et al. 2012; Perelo 2010). In order to monitor and control this engineered microbial structures in waste water treatment system, it is necessary to understand the relationships between the microbial community structure and the process performance. The application of molecular techniques in studies of engineered environmental systems like WWTP and bioreactors has increased the sight into the vast diversity and interaction of microorganisms present in wastewater treatment system (Wojnowska-Bary?a et al. 2010). Microorganisms relevant to bioremediation have been determined by different MTBs based works, such as methanotrophs, related to methane oxidation, certain α- and γ-proteobacteria, related to aerobic hydrocarbon degradation, phylotypes related to *Synothropus* as relevant to anaerobic hydrocarbon degradation, and PAH-degrading bacteria (eg. *Burkholderias, Sphingomonas* and *Mycobacterium*) (Watanabe 2001).

1.7.3. COMMON MTBS FOR CHARACTERIZATION OF BIOREACTORS

Molecular methods, both qualitative and quantitative, have become important in hydrogen-fermenting community analyses to reveal the complexity of the community and to monitor the changes and interactions in the communities during bioprocesses (Tolvanen and Karp 2011). In this subsection a brief explanation of their principles, and their pros and cons, will be exposed.

MBTs employed in this work are related the clone library method (also known as cloning-sequencing method). This is the most widely used method to analyze PCR products amplified from a sample. It consists on the cloning of the multiplied nucleotide chains and then the sequencing of the individual gene fragments (DeSantis et al. 2007). The obtained sequences are compared to known sequences in online databases. Two of this databases are worth to be mentioned: GenBank, from the National Center for Biotechnology Information (NCBI, USA,(Wheeler et al. 2005)) and the Ribosomal Database Project (RDP), from the University of Michigan (Cole et al. 2002)). Both outstand for their vast content in publicly available DNA and RNA sequences of hundreds of thousands organisms, obtained primarily through submissions from individual laboratories and batch submissions from large-scale sequencing projects. Besides they include a variety of features and online software, easy to use, for providing sequence similarity searches on the, databases. The main drawback of this technique is the fact that although clone libraries of 16S rRNA genes permit an initial survey of diversity and identify novel taxa, studies have shown that environmental samples like soil may require over 40,000 clones to document 50% if the richness. Typical clone libraries of 16S rRNA genes contain fewer than 1,000 sequences and therefore reveal only a small portion of the microbial diversity present in a sample. Despite its limitations (e.g., labor intensive, time-consuming, and cost factor (Tolvanen and Karp 2011)), clone libraries are still considered the “gold standard” for preliminary microbial diversity surveys (DeSantis et al. 2007). With the advent of newer and inexpensive sequencing methods, great progress is expected in this method of microbial diversity analysis.

Terminal-restriction fragment length polymorphism (T-RFLP) analysis is a semi-quantitative fingerprinting method used for comparative community analysis (Tymensen et al. 2012). It is a PCR based analysis method targeting the 16S rRNA gene. The PCR products are tagged by using labeled primers and after amplification the amplicons are digested with restriction enzymes, resulting in the production of fluorescent labeled terminal-restriction fragments (T-RFs) (Tymensen et al. 2012). These fragments containing the labels are detected using capillary electrophoresis producing a fingerprint of the microbial community in which the number of different peaks indicates present T-RFs, while the height and area of the peaks indicate the number of their relative abundance. Since each species will in theory be represented by a different T-RF, this method provides a unique profile depending on the species composition of the sample community (Tymensen et al. 2012) .This method is sensitive (limit of sensitivity approximately 0.5% of the community), suitable for high throughput analyses, and the specificity can be tuned by selecting primers of suitable taxonomic resolution amplifying groups or individual species (Tolvanen and Karp 2011), but the biggest drawback is the need for restriction digestion and the problems arising from incomplete digestions (Liu et al. 1997).

Fluorescence in situ hybridization (FISH) is a technique where labeled probes are introduced to cells to bind a precise target in ribosomes that allows researchers to locate the positions of specific DNA sequences on chromosomes (O'Connor 2008). In FISH cells are first fixed to stabilize the morphology and the cell membrane is permeabilized for probe hybridization. The probe is incubated with the sample to allow it to diffuse to the intracellular targets and form sequence specific hybrids. After that the excess probe is washed away and the sample is ready to be analyzed by epifluorescence microscopy or fluorescence-activated cell sorting. Automated image analysis can greatly enhance sample throughput. Digital images can be automatically color calibrated and the cells can be counted and measured (Tolvanen and Karp 2011).

FISH is suitable for quantitative analysis of the samples; however, since it is time consuming due to hybridization it is not suitable for high throughput community structure monitoring and fingerprinting. This technique also requires some information on the community structure before analysis for the selection of right probes. Other drawback of this method is that the number of ribosomes can vary according to the growth phase or the cell size and small amounts of ribosomes might limit detection of mono-labeled probes. Furthermore, the technique is labor intensive and needs appliances with high cost when considering automated analysis and cell sorting (Tolvanen and Karp 2011).

PCR-denaturing gradient gel electrophoresis (DGGE) is perhaps the most popular technique for biodiversity assessment in bioreactor samples (Choi et al. 2007; Kaushik et al. 2010; Liu et al. 2009; Poggi-Varaldo et al.; Wiszniowski et al. 2011; Zhang et al. 2009). In DGGE the target DNA mixture is amplified with GC clamp primers, the sequences are separated in a polyacrylamide gel, and bands are visualized. Separation of DNA sequences in DGGE is based on the electrophoretic mobility of a partially melted DNA in a polyacrylamide gel containing a linearly increasing gradient of denaturants. The separation is centered on the base composition and melting behavior of the molecule rather than the size. For further characterization of the observed bands they can be excised from the gel and re-amplified for sequencing without first cloning the sequences.

DGGE is an efficient tool in community analysis since it provides a visual image on the changes in the community structure. There are, albite, also some drawbacks. DGGE gels with well resolved bands are difficult to produce from very diverse microbial communities or samples containing low biomass and/or PCR inhibitors, and gel to gel variations can be a problem in comparing the profiles (Spiegelman et al. 2005). DGGE is a technique that allows semi-high throughput of samples, but still remains time consuming due to multiple steps compared to PCR analyses. DGGE is not a closed tube method and if sequence information is required additional steps for band cutting, re-amplification, and sequencing add to the time and labor demands of the method (Tolvanen and Karp 2011).

Quantitative PCR (qPCR) is the most sensitive detection method available for nucleic acid targets, when appropriate amplification primers can be designed. In qPCR the fluorescence, which is specific for the amplified target, is measured continuously during PCR reaction. The initial amount of target DNA extracted from a sample is inversely proportional to the threshold cycle determined as a function of background fluorescence and is plotted at a point in which the signal generated from a sample is significantly greater than the background fluorescence. Absolute quantification of the target requires the use of standard dilution series of known concentration of the target DNA. Standards can be either genomic DNA from pure cultures, plasmids carrying a copy of the target gene, or a PCR amplified target DNA segment. Standards are amplified along with the samples and the standard curve is generated from the cycles when the standards cross the threshold value. The sample threshold cycles are compared to the standard curve and the target amount in samples is determined (Tolvanen and Karp 2011).

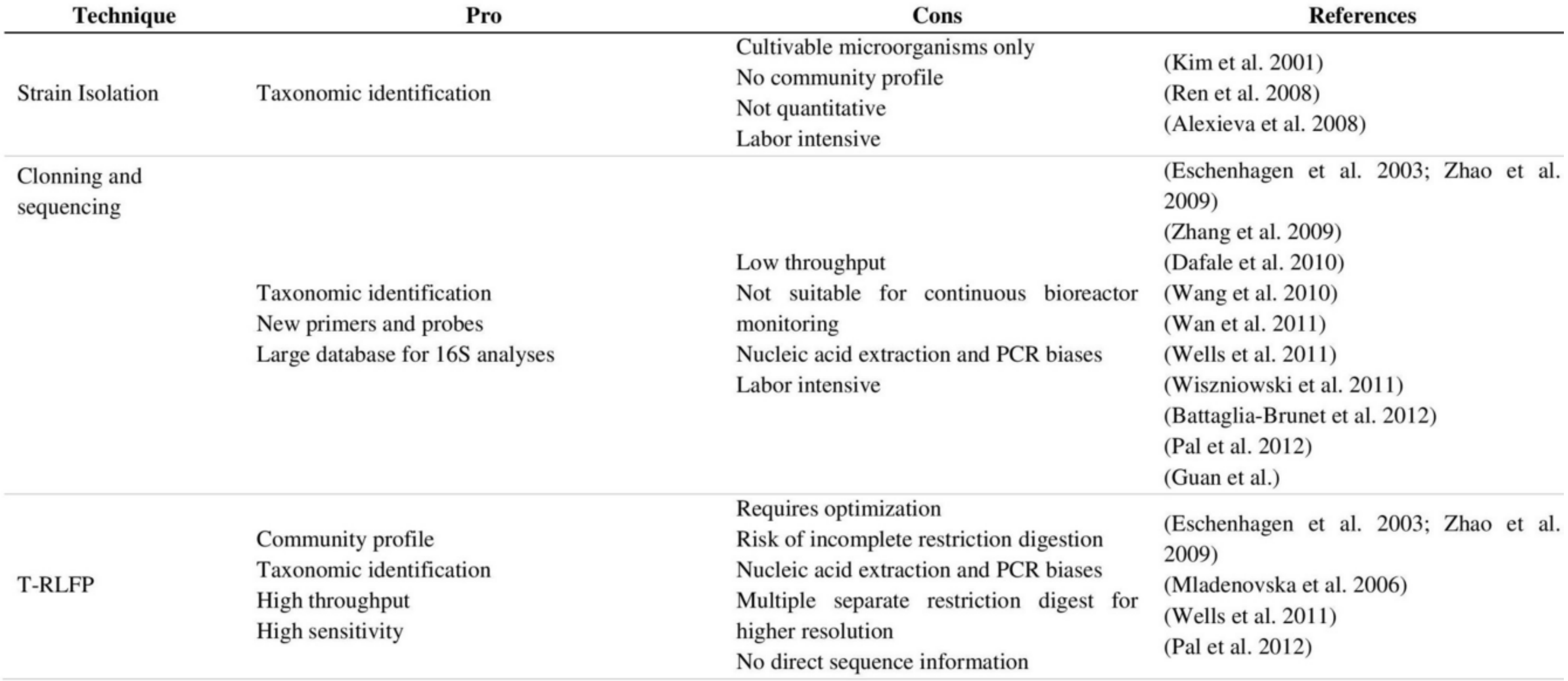
QPCR suffers mostly from PCR associated problems, such as amplification biases, and variations in the nucleic acid extraction step (Talbot et al. 2008). It can, however, be used when large sample quantities need to be analyzed, it is fast and easy to perform with automated machines, and it eliminates the need for post-PCR target analysis

Melting curve analysis is performed after PCR amplification of a target sequence. In this method the amplified DNA is gradually heated and the fluorescence signal coming from dsDNA binding dye, such as SYBR Green, is measured. When the double stranded products dissociate (melt) there is a sudden drop in the fluorescence readings. The temperature of dissociation is the melting temperature of the product and depends both on the length and composition of the PCR product.

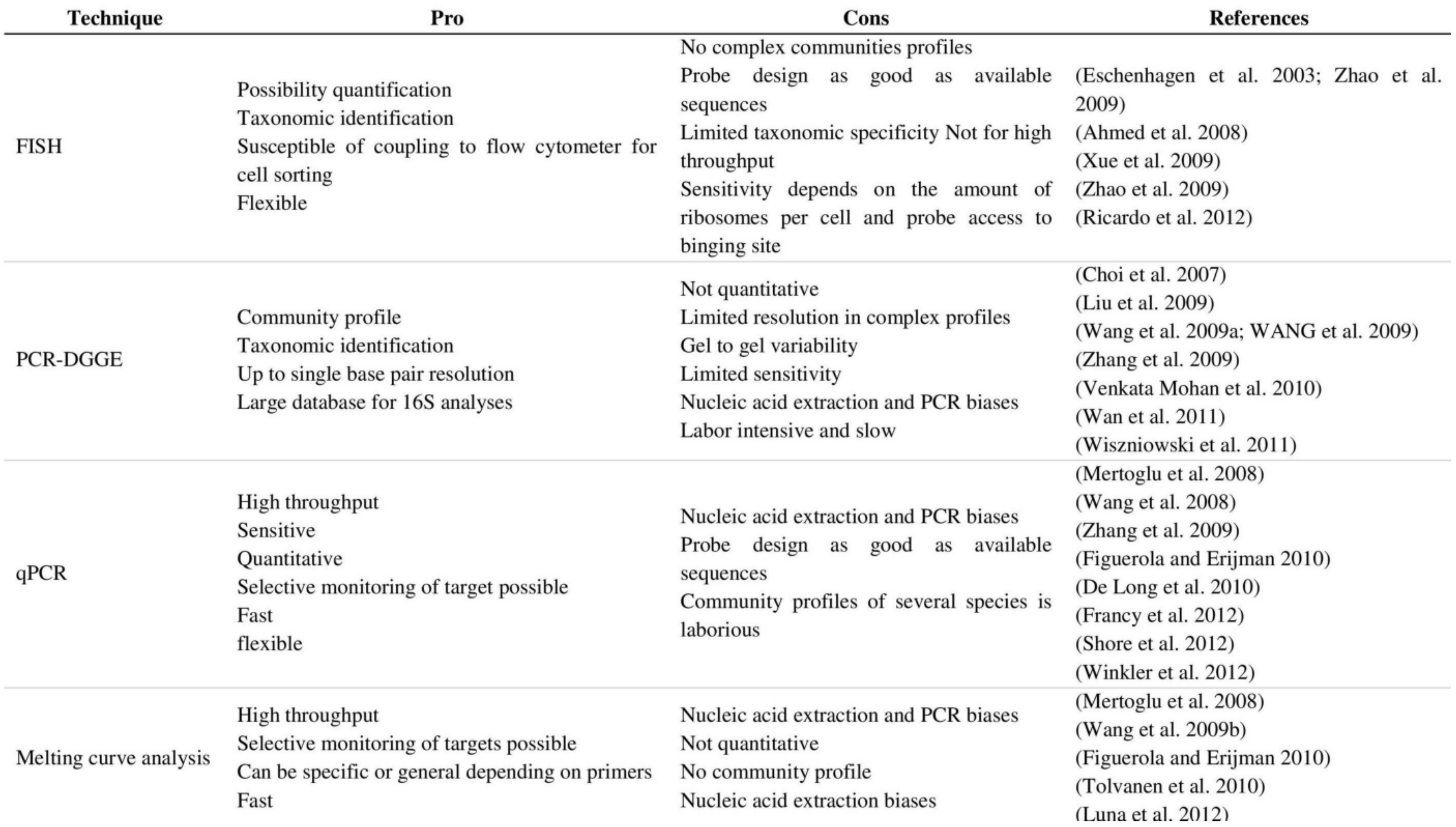
Similar to DGGE analysis, the melting curve method is an end point method and therefore not quantitative. In DGGE the number of bands in lanes can be used to qualitatively estimate the number of targets in one sample and a similar qualitative analysis can be done using melting curves by observing number of melting peaks or changes in peak widths when melting temperatures differ only slightly. It has been reported, that products differing by less than 2 °C in melting temperature can be separated [85]. In melting curve analysis the sequences are not separated and therefore cannot be separately sequenced. Melting curve analysis is, however, faster, closed tube method which can be automatically done with most quantitative PCR machines. It therefore can provide an alternative in the future for faster analysis and easier performance compared to PCR-DGGE

Pros and cons of the cited technologies are summarized in table 17.

Table 17: Microbial community characterization methods used for bioreactors and their advantages and disadvantages (adapted from (Tolvanen and Karp 2011), updated references by the author).



Technique	Pro	Cons	References
Strain Isolation	Taxonomic identification	Cultivable microorganisms only No community profile Not quantitative Labor intensive	(Kim et al. 2001) (Ren et al. 2008) (Alexieva et al. 2008)
Clonning and sequencing	Taxonomic identification New primers and probes Large database for 16S analyses	Low throughput Not suitable for continuous bioreactor monitoring Nucleic acid extraction and PCR biases Labor intensive	(Eschenhagen et al. 2003; Zhao et al. 2009) (Zhang et al. 2009) (Dafale et al. 2010) (Wang et al. 2010) (Wan et al. 2011) (Wells et al. 2011) (Wiszniowski et al. 2011) (Battaglia-Brunet et al. 2012) (Pal et al. 2012) (Guan et al.)
T-RLFP	Community profile Taxonomic identification High throughput High sensitivity	Requires optimization Risk of incomplete restriction digestion Nucleic acid extraction and PCR biases Multiple separate restriction digest for higher resolution No direct sequence information	(Eschenhagen et al. 2003; Zhao et al. 2009) (Mladenovska et al. 2006) (Wells et al. 2011) (Pal et al. 2012)



Technique	Pro	Cons	References
FISH	Possibility quantification Taxonomic identification Susceptible of coupling to flow cytometer for cell sorting Flexible	No complex communities profiles Probe design as good as available sequences Limited taxonomic specificity Not for high throughput Sensitivity depends on the amount of ribosomes per cell and probe access to binging site	(Eschenhagen et al. 2003; Zhao et al. 2009) (Ahmed et al. 2008) (Xue et al. 2009) (Zhao et al. 2009) (Ricardo et al. 2012)
PCR-DGGE	Community profile Taxonomic identification Up to single base pair resolution Large database for 16S analyses	Not quantitative Limited resolution in complex profiles Gel to gel variability Limited sensitivity Nucleic acid extraction and PCR biases Labor intensive and slow	(Choi et al. 2007) (Liu et al. 2009) (Wang et al. 2009a; WANG et al. 2009) (Zhang et al. 2009) (Venkata Mohan et al. 2010) (Wan et al. 2011) (Wiszniowski et al. 2011)
qPCR	High throughput Sensitive Quantitative Selective monitoring of target possible Fast flexible	Nucleic acid extraction and PCR biases Probe design as good as available sequences Community profiles of several species is laborious	(Mertoglu et al. 2008) (Wang et al. 2008) (Zhang et al. 2009) (Figuerola and Erijman 2010) (De Long et al. 2010) (Francy et al. 2012) (Shore et al. 2012) (Winkler et al. 2012)
Melting curve analysis	High throughput Selective monitoring of targets possible Can be specific or general depending on primers Fast	Nucleic acid extraction and PCR biases Not quantitative No community profile Nucleic acid extraction biases	(Mertoglu et al. 2008) (Wang et al. 2009b) (Figuerola and Erijman 2010) (Tolvanen et al. 2010) (Luna et al. 2012)

1.7.4. KEY ISSUES AND NEW TRENDS IN MOLECULAR BIOLOGY TECHNIQUES

Nowadays mainly molecular techniques and combination of these techniques are used in scientific research, but most are not used in the field. To date, PCR, qPCR are the only mentioned MTBs that are used by practitioners on a somewhat regular basis. In the case of bioremediation, the current state of the practice is quite empirical, MBTs could reduce this uncertainty. However, there are still some goals that need to be achieved for spreading their use by professionals. On one hand MBT development should incorporate the regulatory community into the process to maximize appropriate use of tools. Government agencies may develop a “technology overview” document outlining the “state of the science” for these methods, what they could be useful for now, what is on the horizon, etc. On the other hand, the creation of standardized analytical methods is also peremptory. Although analytical methods are constantly being optimized, there are significant elements of the procedures that could be generalized to help mitigate concern that analyses between laboratories are not comparable (Alleman et al. 2005).

Together with functional microbial ecology, that will link community structure and function, and postgenomic techniques (metaproteomics and proteogenomic), next-generation DNA sequencing techniques will transform the field. The capability of large scale sequencing techniques to generate billions of reads at low cost with high speed will be useful in many applications such as whole-genome sequencing, metagenomics, metatranscriptomics, and proteogenomics. Pyrosequencing also would take advantage of these new sequencing technologies.

Pyrosequencing allows multiple samples to be combined in a single run, and after sequencing, the reads can be parsed through their assigned nucleotide barcode, which is added in templates during PCR (Rastogi and Sani 2011). The results, composed by millions of sequences, can be compared with the previous named sequences databases obtaining extremely detailed composition of the bacterial population. Enormous phylogenetic diversity found in soil, water or wastewater samples could only be appreciated in its entirety by this technique.

1.8. EXPERIMENTAL DESIGN

Many phenomena studied in science and engineering can be explained, at least superficially, by means of easy physical mechanisms that can be defined mathematically by what is called a *mechanistic model* (e.g. pressure under a water column of a certain height: $P = \rho g h$). These equations can be used for predicting the results of the phenomena without the need of doing an experimental test. However, for more complicate phenomena or a deeper study of simpler cases it is extremely difficult to find the equation to define the system. In those circumstances it is only possible to elucidate a partial mathematical model that defines the system just under certain conditions, which can develop what is known as *empirical model.*

Experimental design consists on a series of criteria that enables to identify the essential experiments (not more, not less) to obtain quality information to develop an empirical model that allows modelling the studied phenomenon in a certain range of conditions, with an acceptable reliability (based on statistical considerations) and reducing the experimental effort (Leardi 2009). The main steps to apply experimental design for a certain study are summarized in table 18.

Table 18. Main steps for experimental design

Basic steps for Experimental Design
1. Statement of the problem
2. Selection of the response variable to study
3. Selection of the factors and the range of variation of these values
4. Choice of experimental design type
5. Conducting experiments plan
6. Statistical analysis of the results
7. Conclusions and empirical model

Depending on the system and the problem, and the number of experiments that can be done (according to budget and operational considerations), and taking into account that the quality of the results depends on the distribution of the experiments in the experimental domain (Leardi 2009); different experimental design types can be used. Table 19 shows a short list of different types of experiments design strategies depending on their main features.

Table 19. Experiments design strategies

	Main feature
Comparative experiments	Single variable experiments with only two levels
Variance analysis	Single variable experiments with more than two levels
Randomized blocks, Latin squares and similar	Multivariate experiments that allows eliminating the effect of those variables which are known and out of control of the experimenter
Factorial, fractioned factorial and similar	Multivariate experiments that study the effect of two factors or more and their interaction

In most of the systems and circumstances, the studied phenomenon is affected by more than one variable at the same time. Therefore, in opposition to the traditional an ineffective methodology of *one variable at a time*, OVAT, in which only one factor is changed in every experiment (and it is supposed that the starring variables are independent from each other (Leardi 2009)), experimental design criteria impose the use of multivariate approach. This strategy implies the change of more than one factor in each experiment, and allows scheduling an affordable number of experiments, even in the case there are numerous variables taking part. Furthermore some experimental design strategies are able to determine the existence of interactions between factors, such as factorial designs and similar.

When there is the need to optimize the influence of the different factors in a certain response variable, a modification of the factorial method, that will increase the number of experiments, enables the use of the *response surface methodology*, RSM. The surface will be defined by the mathematical model derived from the statistical processing of the results of the performed experiments according to a certain experimental design strategy (Box and Wilson 1951). Equation 1 is the general expression of this model:

$$y = (x_1, x_2, x_3 \ldots, x_k) = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} {x_i}^2 + \sum \sum_{i \neq j} \beta_{ij} x_i x_j \qquad \text{eq. } 1$$

It constitutes a hyper-plane like the one shown in fig. 20.

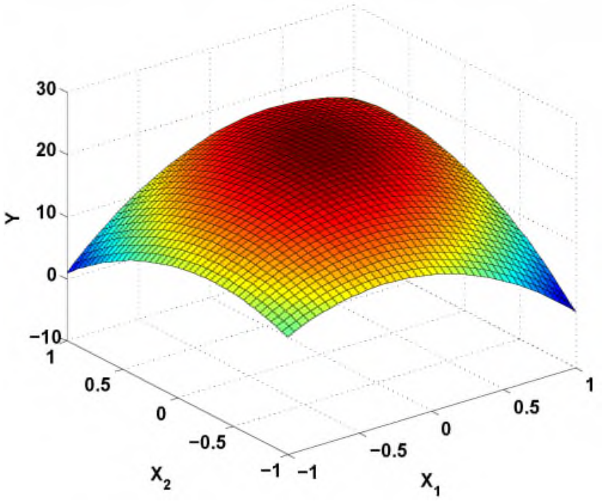


Figure 20. Example of response surface.

By looking at this plot, the general behavior of the phenomenon can be easily understood (Leardi 2009). They can be used to study qualitatively the effect of differing operating conditions, locate the optimum, and estimate a mathematical equation that describes the system. The curvature in a certain experimental range usually indicates that the optimum point is close and the phenomenon is strongly influenced by the involved variables. When the number of dimensions exceeds 3, parallel axes can be used to represent hyper-dimensional figures (Gennings et al. 1990) or several 3 dimension plots can be used (Grossman 1986).

1.8.1. CENTERED COMPOSITE DESIGN

The factorial design is a kind of experiment planning strategy that it is used when studying the effect on some response on several factors at the same time, taking into account both the individual effects and interactions that may occur. Usually, rounds of experiments are conducted wherein all variables take two extreme values (there are experiment plans with more than two levels for each factor). The number of conducted experiments is determined by 2^k, where k is the number of factors to evaluate.

The results of a factorial experiment can be analyzed using ANOVA test or *regression analysis*. This analysis easy estimates the relative main effect for a factor and the interactions among them. Depending on the number of factors involved, a linear mathematical model can be determined based on those effects, as can be seen in table 20.

Table 20. Mathematical models that can be obtaining depending the number of factors in the experimental design

#factors	**#experiments**	**Mathematical model**
2	$2^2=4$	$y=\beta_0+\beta_1x_1+\beta_2x_2+\beta_{12}x_1x_2$
3	$2^3=8$	$y=\beta_0+\beta_1x_1+\beta_2x_2+\beta_3x_3+\beta_{12}x_1x_2+\beta_{13}x_1x_3+\beta_{23}x_2x_3+\beta_{123}x_1x_2x_3$
k	2^k	$y=\beta_0+\beta_1x_1+\cdots+\beta_kx_k+\beta_{12}x_1x_2+\cdots+\beta_{1k}x_1x_{k+}+\beta_{23}x_2x_3+\cdots+\beta_{2k}x_2x_k+\cdots+\beta_{k-1k}x_{k-1}x_k+\beta_{123}x_1x_2x_3+\cdots$

However, in order to optimize the response by means of RSM, and in comparison to eq. 1, second order terms are required on the mathematical model. For this purpose some experiments, 2k, have to be added to the 2^k factorial plan, and n centered experiments; this composes what is called as *Centered Composite Design.*

Those 2k new experiments are considered as *axial points*. Thus, in a system with two variables, 4 extreme points and as many centered experiments as possible (depending on budget and time), together with 4 factorial experiments are depicted in fig. 21, where blue dots are factorial points, red dots are axial points and the yellow dot represents central point.

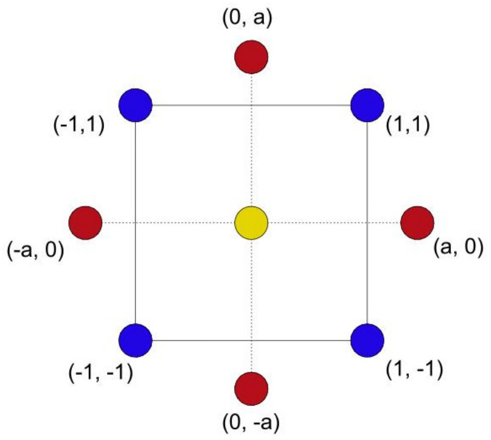


Figure 21: Central Composite Design for k=2. This squeme represents a normalized general case. 1 and -1 enconde the highest and lowest level of each factor. 0 represents the intermediate point between two levels. a denotes a certain value equal or above the highest level.

After determining the levels of each factor, two other parameters have to be specified in this model: the distance of axial points and the number of repetitions of the center point. While the latter is chosen by operational criteria, a can be defined as $a=\sqrt{k}$, as it is the case of Spherical Centered Composite Design; or as $a=1$, Face Centered Composite Design. This latter design is particularly useful in cases in which the system does not allow the factors take values outside the range of its two extremes. Fig. 22 represents these two systems for three factors.

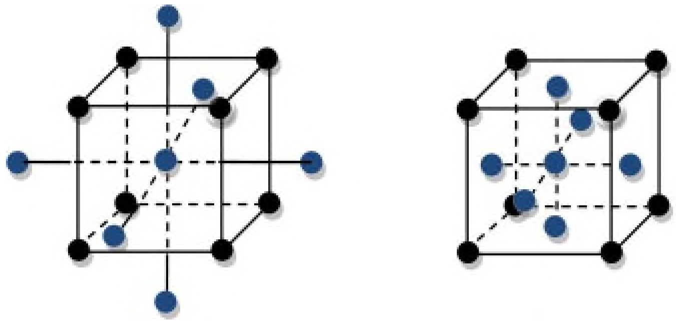


Figure 22: Spherical Centered Composite Design and Face Centered Composite Design, both for three variables.