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INFLUENCE OF ALTERNATIVE ELECTRON ACCEPTORS ON THE ANAEROBIC BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

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Abstract

Polycyclic aromatic hydrocarbons are chemicals produced by both human activities and natural sources and they have been present in the biosphere since millions of years. For this reason microorganisms should have developed, during the world history, the capacity of metabolized them under different electron acceptors and redox conditions. The deep understanding of these natural attenuation processes and of microbial degradation pathways has a main importance in the cleanup of contaminated areas. Anaerobic degradation of aromatic hydrocarbons is often presumed to be slow and of a minor ecological significance compared with the aerobic processes; however anaerobic bioremediation may play a key role in the transformation of organic pollutants when oxygen demand exceeds supply in natural environments. Under such conditions, anoxic and anaerobic degradation mediated by denitrifying or sulphate-reducing bacteria can become a key pathway for the contaminated lands clean up.

Actually not much is known about anaerobic bioremediation processes. Anaerobic biodegrading techniques may be really interesting for the future, because they give the possibility of treating contaminated soil directly in their natural status, decreasing the costs concerning the oxygen supply, which usually are the highest ones, and about soil excavations and transports in appropriate sites for a further disposal.

The aim of this dissertation work is to characterize the conditions favouring the anaerobic degradation of polycyclic aromatic hydrocarbons. Special focus will be given to the assessment of the various AEA efficiency, the characterization of degradation performance and rates under different redox conditions as well as toxicity monitoring. A comparison with aerobic and anaerobic degradation concerning the same contaminated material is also made to estimate the different biodegradation times.

Chapter 1
State of scientific and technical knowledge

1.1 Contaminated sites situation

Contaminated sites usually contain different pollutants which can constitute a risk to the health of human, animals and generally to the environment. Although, in the last years, several improvements have been made to reduce the dangerous and toxic loss in the industrial areas, major releases still occur: lots of contaminated sites are present all over the world and new ones are continually discovered.

The sources of contamination can be really different, like chemicals and petrochemicals industries, but also fuel stations, mining activities, landfills, agricultural runoff, soil exploitation and airborne particles.

The European Environmental Agency have made an estimation of contaminated sites all over the Europe and the result is that more than 3 millions potentially polluted sites are present, and the 8% of these are already contaminated (more than 240,000 sites) and need a land reclamation (EEA, 2007). The principal causes of the contamination are industrial production and commercial service, oil industries and waste treatments and disposals. Concerning the kind of contamination, the 13% is covered by polycyclic aromatic hydrocarbons, even though the main contaminations come from heavy metal and mineral oils.

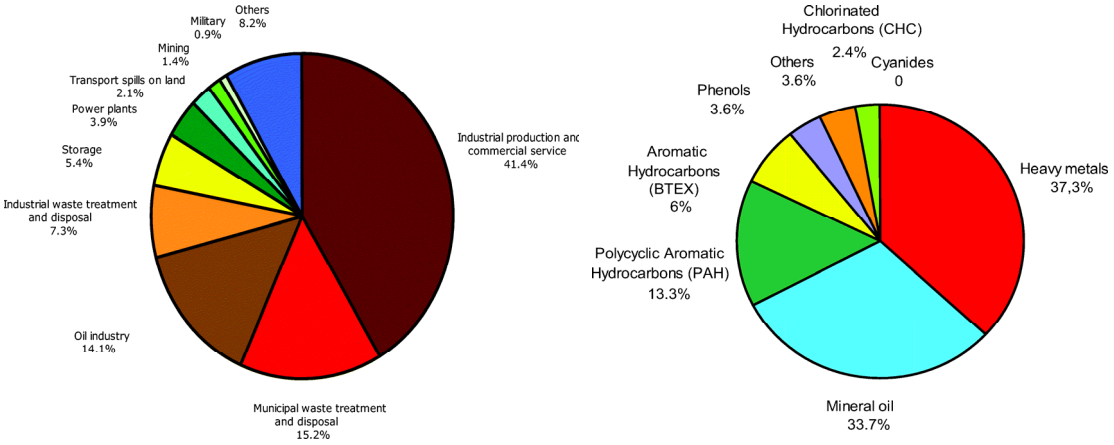


Figure 1.1 – Main sources and kinds of contaminated sites in Europe (EEA, 2007)

Actually in Austria more than 50000 old deposits and contaminated sites are detected by the Austrian environmental federal office (Siller and Weihs, 2008), concerning old deposits and old industries sites; in addition there are more then 2,000 potentially contaminated areas, which are monitored. In the following tables is possible to see the national distribution of these polluted areas.

Contaminated Sites			
Federal province	**Old deposits**	**Contaminated sites**	**Sum**
Burgenland	99	3,098	3,179
Carinthia	473	2,455	2,928
Lower Austria	1,169	2,385	3,527
Upper Austria	1,465	9,099	10,564
Salzburg	416	5,603	6,019
Styria	375	4,301	4,676
Tyrol	646	4,664	5,310
Vorarlberg	14	6	20
Vienna	341	14,499	14,840
Sum	**4,998**	**46,083**	**51,081**

Data from "Verdachtsflächenkataster und Altlastenatlas" UBA, 2008

Potentially Contaminated Areas			
Federal province	**Old deposits**	**Contaminated sites**	**Sum**
Burgenland	38	2	40
Carinthia	35	12	47
Lower Austria	451	52	503
Upper Austria	641	115	756
Salzburg	124	174	298
Styria	211	15	226
Tyrol	99	5	104
Vorarlberg	9	3	12
Vienna	42	11	53
Sum	**1,650**	**389**	**2,039**

Data from "Verdachtsflächenkataster und Altlastenatlas" UBA, 2008

Table 1.1 – Austrian contaminated areas

Below a picture reports the spatial distribution of these contaminated sites.

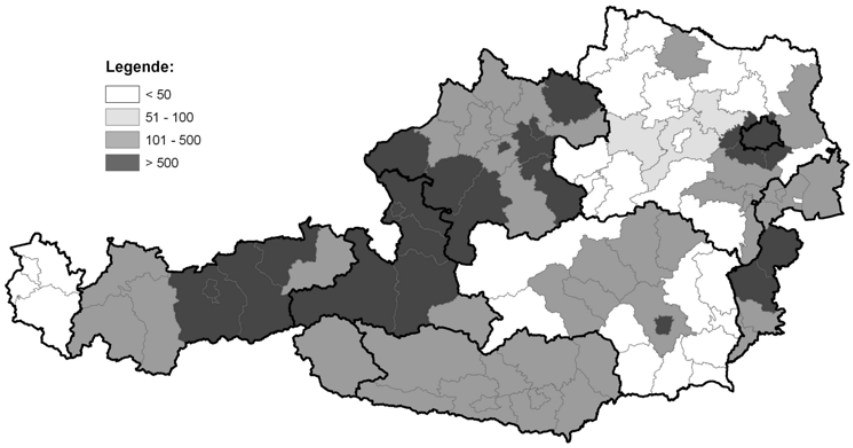


Figure 1.2 – Territorial distribution of contaminated areas in Austria

As it is possible to see in the following graph, about 30% of all contaminated sites is contaminated with polycyclic aromatic hydrocarbons (PAH), which represent besides chlorinated hydrocarbons (CHC) and mineral oil hydrocarbons, the major organic pollutants.

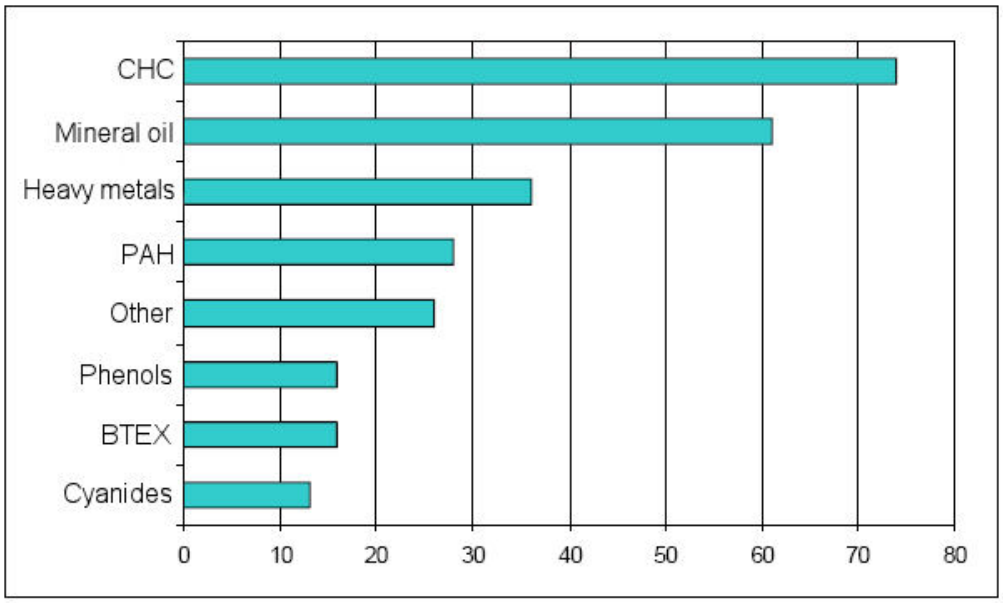


Figure 1.3 – Different kind of contaminations in the Austrian polluted sites

About the Italian situation, more than 16000 potentially polluted sites are registered by the national government and more than 4000 are really polluted and need remediation actions. Through successive laws (d.lgs 462/98, d.lgs 388/00, d.lgs 468/01, d.lgs 179/02) about 50 of the previously mentioned areas are classified as "sites of national interest" for their large dimension and extensive contamination. The costs estimated for their management are about 3,150 billions of euro but the government founding covered just the 18% of the required budget, about 550 millions euro (Fava, 2008). The inferior number of polluted sites has to be connected not to a higher level of environmental consciousness, but to a delay, from the Italian governance, in detecting contaminated areas (Ciafani, 2006). The territorial distribution of these sites is focused on the northern part, where is placed the 40% of the whole Italian contaminated sites, followed by the central part with the 27%, the southern part with the 22% and the islands with the 11% of the total sites amount. The following figure shows the territorial distribution of the 50 sites of national interest in the Italian territory.

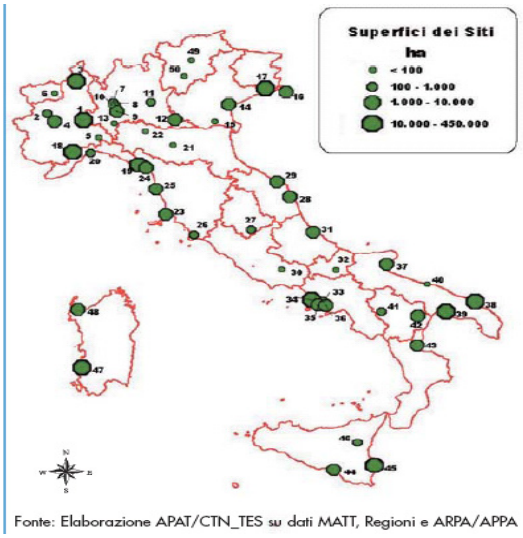


Figure 1.4 – Territorial distribution of Italian sites of national interest

Due to the provided data, nowadays, the development of methods to clean up polluted sites is a main need, otherwise many of these sites could become a source of drinking water contamination, constituting a health hazard for current and future generations. Primarily due to the cost and time consideration, physical and chemical treatment processes are the most widely used remediation methods. Nevertheless the biological degradation is growing up and taking an establish place as a soil restoration technology.

1.2 Polycyclic Aromatic Hydrocarbons

1.2.1 Environmental occurrence

Nowadays, environment is widely contaminated by hydrocarbons due to different causes. Although the aliphatic or monoaromatic hydrocarbons are mostly degraded naturally and quickly by environmental microorganisms, polycyclic compounds, that are less volatile and have a high affinity for particulate matter, are often deposit in the bottom sediments, persisting for long time, till they are degraded, re-suspended, bio-accumulated, or remove by dredging, representing an environmental trouble.

Polycyclic Aromatic Hydrocarbons, also named polyarenes or PAHs, are among the most widely distributed carcinogenic compounds in the environment. Principal

sources of this contaminant are combustion of fossil fuel in energy generation, incineration of waste and coke production. Especially in urban environments of industrialized countries, vehicles are another important source of PAHs emission, with areas close to highways and airports predominantly affected. Besides these anthropogenic sources, PAHs are also naturally produced as a consequence of forest fires and volcanic activities. However their contribution to the overall emission is negligible.

Due to their ubiquitous distribution, PAHs may be found in all compartments: air, water, soil and also in consumer products and food, exposing all biological receptors, including human beings. Concerning soils, typical concentrations of carcinogenic PAHs range are from 5-100 ppm; however higher concentrations of PAHs are found in soils near anthropogenic source, such as highways or industries. Not surprisingly, urban soils contained elevated concentrations of PAHs, ranging from 600-3000 ppm (Szolar, 2002). Another author (Harvey, 1997) reports that significant levels of polyarenes are also found in the soil in all regions of the Earth. Usually the urban zones concentration is ten to hundred times higher than those in less populous and undeveloped areas.

In addition, this kind of compounds is lipophilic, so that they mix easier with oil than with water, and little water soluble and volatile. Because of these reasons PAHs in the environment are found primarily in soil, sediment and oily substances, as opposed to water or air. In particular they are found on various contaminated sites such as former gas plant sites, mineral oil refineries (Coates et al.,1995) and also on marine and coastal sediments near urban and industrial cities (Chang et al., 2008).

1.2.2 Cercla priority list of hazardous substances

The number of possible PAH compounds that could be obtained by methyl group substitution could exceed comprehension. For this reason, only a minuscule percentage of the theoretically possible substituted derivates are likely to be deeply investigated. The agency for Toxic Substances and Disease Registry (USA) had listed 16 PAHs, that are considered the most dangerous and carcinogenic of this family of organic compounds, as priority pollutants (*Cercla* Priority List of Hazardous Substances). The United States Environmental Protection Agency (US-

EPA) has characterized them and for this reason they are known as 16 EPA PAHs. A list of them is reported below.

- Naphthalene
- Acenaphthylene
- Acenaphtene
- Fluorene
- Phenanthrene
- Anthracene
- Fluoranthene
- Pyrene
- Benz(*a*)anthracene
- Chrysene
- Benzo(*a*)pyrene
- Benzo(*k*)fluoranthene
- Benzo(*b*)fluoranthene
- Dibenzo(*a*,*h*)anthracene
- Benzo(*g*,*h*,*i*)perylene
- Indeno(1,2,3-c,d)pyrene

In the following picture the chemical structure of these 16 EPA PAHs is reported.

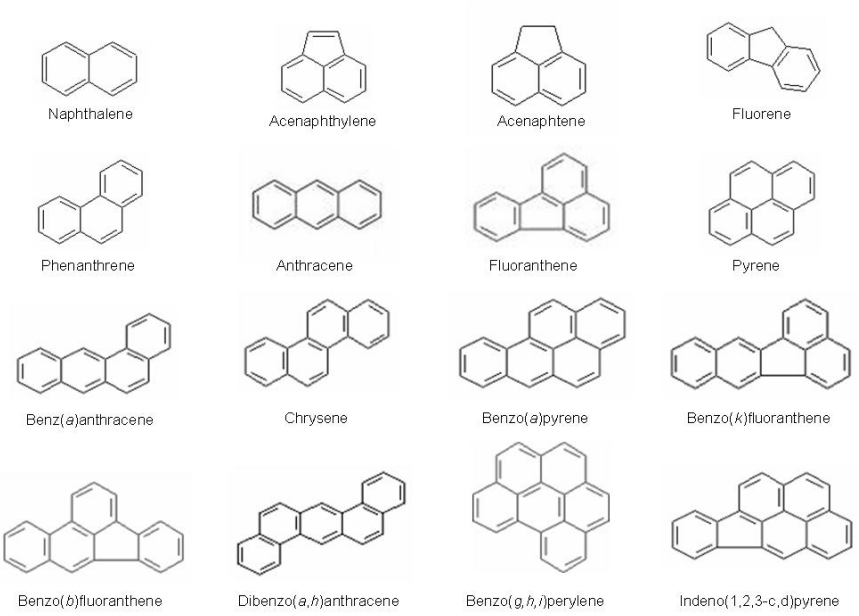


Figure 1.5 – Chemical structures of 16 priority PAHs by CERCLA Priority List of Hazardous Substances

In usual analysis of polluted materials, these 16 organic compounds are investigated. In the experiments that will be further presented, 5 of these chemicals are closely investigated.

1.2.3 Chemical properties

PAHs are composed of fused aromatic rings whose biochemical persistence arise from dense clouds of π-electrons on both sides of the ring structure, making them resistant to the nucleophilic attacks. They are usually solid at room temperature, and have usually high values of boiling and melting point. The vapor pressure of these compounds is usually low and is also inversely proportional to the number of aromatic rings (Johnsen et al., 2005).

This class of compounds is very persistent in environment. This persistence is dependent on a variety of factors, such as the chemical structure, the concentration and dispersion of PAH and the bioavailability of the contaminant. Generally, the higher the molecular weight of the PAH molecule, the higher the hydrophobicity and toxicity, the longer the environmental persistence of the molecule (Bamfort and Singleton, 2005).

Concerning stability, for the series of linearly-fused polyarenes, such as naphthalene and anthracene, stability decreases as the number of rings increases; each member becomes more like olefin and less aromatic in character than its predecessors. On the other hands, in the series of angularly-fused polyarenes, such as phenanthrene and chrysene stability doesn't decrease with the addition of aromatic rings.

Concerning solubility, PAHs show the tendency to decrease with increase in molecular size. However solubility depends also from molecular symmetry, planarity and presence of substituents. PAHs that are planar and symmetrical are able to pack closely, so that the energy required for the solubilization tends to be relatively high, therefore compounds tend to be less soluble. Conversely, PAHs that lack symmetry, planarity and possess substituents are often reasonably soluble in organic media (Harvey, 1997). The solubility is also influenced by the temperature (Barkay et al., 1999). Moreover, PAHs are extremely lipophilic compounds. A possible measure of the lipophilicity of PAHs is given by partitioning coefficient of water/n-octanol phase, K_{ow}, which express the compounds capacity of build up a-polar phases, such as adipose tissues of organisms. The formula used for the calculation is:

$$K_{ow} = [S]_{oc} \Big/ [S]_{w}$$

where:

$[S]_{oc}$ = concentration of S substance in octanol express in M or ppm;

$[S]_{w}$ = concentration of S substance in water express in M or ppm.

Usually it is more employed the $\log(K_{ow})$, and US-EPA states that compounds which have a value of $\log(K_{ow})$ greater than 3,5 must be considered potentially dangerous for the environment (Lopez, 2002).

Another important parameter, regarding likewise solubility, is K_{oc}, the ratio of the chemical adsorbed per unit weight of organic carbon in the soil or sediment to the concentration of the chemical in solution at equilibrium. Usually, the higher this ration is, the lower water solubility.

In the table 1.2 are listed some of most relevant PAHs characteristics; water solubility is reported at room temperature (Loibner and Farinosa, 2003).

Compound	MW	Log K_{ow}	K_{oc} (cm^3/g)	Water Solubility (µg/L)
Naphthalene	128,2	3,4 [c]	$2,0*10^3$	31500 [a]
Acenaphthene	154,2	3,9 [c]	$7,1*10^3$	2700 [a]
Acenaphthylene	152,2	4,1 [b]	$4,8*10^3$	14400 [a]
Fluorene	166,2	4,2 [c]	$1,4*10^4$	1400 [a]
Anthracene	178,2	4,5 [b]	$3,0*10^4$	73 [b]
Phenanthrene	178,2	4,6 [c]	$2,3*10^4$	890 [a]
Pyrene	202,2	4,9 [b]	$1,1*10^5$	135 [b]
Fluoranthene	202,2	5,1 [c]	$1,1*10^5$	260 [b]
Benzo(a)anthracene	228,3	5,7 [c]	$4*10^5$	14 [b]
Chrysene	228,3	5,8 [b]	$4*10^5$	2,0 [b]
Benzo(a)pyrene	252,3	6,2 [b]	$1*10^6$	3,8 [b]
Benzo(b)Fluoranthene	252,3	6,4 [b]	$1,2*10^6$	1,5 [b]
Benzo(k)Fluoranthene	252,3	6,4 [b]	$1,2*10^6$	0,8 [b]
Dibenzo(a,h)anthracene	278,4	6,5 [b]	$3,8*10^6$	0,6 [b]
Indeno(1,2,3-c,d)pyrene	276,3	6,7 [c]	$3,5*10^6$	0,19 [b]
Benzo(g,h,i)Perylene	276,3	7,1 [c]	$7,8*10^6$	0,26 [c]

) [a] Own measurement) [b] Sverdrup (2001)) [c] BP RISC 4.0

Table 1.2 – 16 EPA PAHs chemical properties

1.2.3.1 Volatilization

An important phenomenon concerning the PAHs removal from soil is the volatilization, that is the process whereby a dissolved substance is vaporized. Through this mechanism, the PAHs decrease in soil is not connected to a biological loss, but to physical processes. As reported by Sun and Weavers (2006), the volatilization of organic compounds is related to their boiling point: the lower is the boiling point, the higher is the volatilization. Hence, low molecular weigh PAHs are more affected by this phenomenon than high molecular weigh PAHs, since their boiling point is lower.

Park et al. (1990) measured the abiotic losses in a PAHs biodegradation process in 2 sandy-loam soils, including also volatilization measurements. They pointed out that volatilization is significant for naphthalene (average removal percentage: 31%) and for 1-methylnaphthalene (average removal percentage 22%), however no significant volatilization has been observed for other PAH compounds. Nevertheless phenanthrene and pyrene volatilization has been reported by Guieysse et al. (2001): the principal abiotic loss in the microbial degradation process in a bioreactor were likely caused by volatilization.

1.2.4 Toxicity

PAHs are highly potent carcinogens that can produce tumors in some organisms at even single doses. Many PAHs display also acute mutagenic and teratogenic properties (Bamforth and Singleton, 2005). Their effects are wide-ranging within an organism and have been found in many types of organisms, including mammals, birds, invertebrates, plants, amphibians, fish, and humans. The rapid adsorption of PAHs by humans results in a high potential for biomagnification in the food chain. Uptake rates are generally governed by PAH concentration, PAH water solubility, soil type, and PAH physicochemical state (vapor or particulate). In general, the grater the number of benzene rings, the greater the toxicity of PAHs.

Mammals can absorb PAHs by inhalation, dermal contact, or ingestion. Plants can absorb PAHs directly from soils through their roots, and translocated them to other plant parts such as developing shoots. Uptake rates are generally governed by PAH concentration, PAH water solubility, soil type, and PAH physicochemical state

(Eisler, 1987). Lower molecular weight (LMW) PAHs are absorbed more readily than higher molecular weight (HMW) PAHs. LMW PAHs have higher volatilization rates and are more readily leached as compared with HMW PAHs.

A dangerous phenomenon that can take place in natural environments is the *bioaccumulation*, that occurs when organisms absorbs a toxic substance at a rate greater than that at which the substance is lost. Thus, the longer the biological half-life of the substance the greater the risk of chronic poisoning, even if environmental levels of the toxin are very low. PAH bioaccumulation has been shown in terrestrial invertebrates and voles; in earthworm, for example, levels have been detected 30-60 times greater than soil concentrations (US-EPA, 2008).

The EPA has classified seven PAH compounds as probable human carcinogens that are benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, dibenz(a,h)anthracene, and indeno(1,2,3-cd)pyrene. Benzo(a)pyrene is one of the most potent chemical carcinogens known, even though it requires, like other same class compounds, a metabolic activation before being able to bind to DNA, RNA and proteins (Sutherland et al., 1995).

The relatively PAHs toxicity can be measured through the LD_{50} (Lethal Dose 50), that is the dose necessary to kill the 50% of the population exposed to the contaminant. It is expressed in milligrams of contaminant per kilogram of the subject's body weigh that can cause the death in the 50% of cases. In table 1.3 the LD_{50} is reported for some principal PAHs, as displayed by Bamforth and Singleton (2005).

PAHs	LD_{50} Value (mg/kg)	Test Subject
Naphthalene	533 – 710	Mice
Phenanthrene	750	Mice
Anthracene	> 430	Mice
Fluoranthene	100	Mice
Pyrene	514	Mice
Benzo(a)pyrene	232	Mice

Table 1.3 – LD_{50} values of some representative PAHs

1.3 Bioremediation Principles

Naturally in soil are present microorganisms which are able to metabolize contaminants under either oxidative or reducing conditions, degrading them into non toxic products, such as water and carbon dioxide or methane and organic acids (US-EPA, 1991). The process of bioremediation refers directly to the stimulation of this natural degradation in different ways, such as adding microorganisms to the soil or providing appropriate conditions or amendments (as oxygen, alternative electron acceptors, nutrients) to favorite the microorganisms growth. Normally the natural process can take many years for the complete degradation: the key is to find out how to accelerate the rate of biodegradation to detoxify the target compounds in a finite time period.

The entire process, both in aerobic and anaerobic conditions, is based on a sequential redox reactions, catalyzed by several enzymes produced by microorganisms, that yield energy for the microorganisms growth (Rockne and Reddy, 2003). Basing on different conditions, different enzymes are involved. Usually oxygen, if it is present, is the favorite electron acceptor, since it provides the maximum energy production, maximizing the microorganisms growth per unit amount of electron donors used. If oxygen is not present, other electron acceptors can be used by different microorganisms, involving a lower growth rate and so a slower contaminants biodegradation.

1.3.1 Terminology

In the following paragraph the meaning of some terms concerning several aspects of bioremediation are elucidated, for a further better understanding.

- **Natural Attenuation**

Natural attenuation includes physical, chemical and biological processes that occur to decrease the beginning contamination level in a soil (van Cauwenberghe and Roote, 1998), that are:

→ *Biodegradation*, due to which indigenous microorganisms, naturally present in the soil, degrade the pollutants through redox reactions;

→ *Chemical transformation*, concerning the contaminants alteration. The transformation rate depends on many factors, including soil, groundwater and chemicals parameters;

→ *Stabilization*, because of pollutants are bounded to the soil by stabilizing agents, avoiding their migration. This process is useful to limit the contamination phenomenon, but decrease the contaminant availability for microorganisms, since the degradation happens mostly in the water phase;

→ *Volatilization*, that concerns the transfer of volatile organic compounds (VOCs) to the vadose zone from the saturated zone;

→ *Dispersion and dilution*, that refer to the increase of ratio of groundwater to the contamination source and the following decrease of contaminants concentrations, assuming that the contamination source is stopped.

This is actually a cheap remediation method, that allows resources to be devoted to other higher priority sites. However the required degradation time is longer than the active remediation one in reaching the remedial targets.

- **Enhanced biodegradation**

This technology is directed towards the microorganisms activity stimulation in using the pollutant as the energy source, by creating suitable conditions for microorganisms. Usually it concerns providing oxygen in case of aerobic degradation, alternative electron acceptors in case of anaerobic conditions and nutrients (Bach et al., 2005). In addition temperature, pH and oxidation reduction potential are important for the process realization; for this reason good conditions for these chemical parameters must be guaranteed.

Usually the “in situ” enhancement is performed through percolation or injection of uncontaminated water with nutrients and dissolved oxygen when it is needed, using injection or extraction wells. The well disposition depends on the area of contamination and the soil permeability (van Cauwenberghe and Roote, 1998).

Due to this stimulation the degradation time is reduced, allowing, when is needed, to reach faster the concentration aim.

- **Bioaugmentation**

This is also an enhanced biodegradation process in which the addition of specific substrate degrading microorganisms is involved, that is useful to stimulate the indigenous microorganisms action. If the indigenous variety is not able to metabolize the organic compounds, exogenous varieties with more sophisticated pathways are introduced to increase the biological capability to remove the pollutants. Usually the specific populations are selected by enrichment culturing or genetic manipulation.

This practice is not always successful, due to many factors: the inoculants activity could be limited by high value of contaminants concentration, by the pH, the salinity of the water phase, and the osmotic and hydrostatic pressure, acting alone or all at the same time. In addition also the subsurface environment could contain chemicals toxic or inhibitors of the inoculated bacteria growth and activity. Finally also the mixing, that guarantees the contact between microorganisms and the substrate, sometimes may be difficult to achieve at many sites (van Cauwenberghe and Roote, 1998).

- **Mineralization and biotransformation**

The results of the microbial activity can be different. The best result you can have is the complete mineralization of contaminants, that, starting from a complex organic molecule and passing through some intermediate metabolites, allows to obtain at the end energy for cells, carbon dioxide, water and inorganic products, such as chlorine, nitrogen and others. This case is the best one since starting from xenobiotic compounds it is possible to get compounds non toxic for the environment (Bezalel et al., 1996).

However it is not always possible to obtain the complete mineralization of the pollutants and sometimes just a biotransformation into usually less toxic or non toxic, due to an incomplete substrate oxidation. In this case starting from a xenobiotic compound it is possible to obtain as product another organic molecule, energy and also carbon dioxide, water and inorganic products. The organic products are structurally simpler and more oxidized than the starting one. Anyway the biotransformation is not always a useful process because it is possible to obtain an organic product that is more toxic or less bioavailable for microorganisms.

Frequently the biotransformation could occur in case of co-metabolism, that means that the pollutant is not the only carbon source (this is the case of "direct metabolism"), but other organic molecules, easier to degrade, are present to support the specific microorganisms growth. In fact if the alternative carbon source finishes before the complete mineralization, it is possible to obtain only a transformation.

- **Bioavailability**

A factor really important for the bioremediation results is the bioavailability, that represents to the accessibility of a chemical for assimilation and possible toxicity (Alexander, 2000). Generally the microorganisms degradation of pollutants depends on 2 factors:

→ The rate of uptake and metabolism;

→ The rate of transfer to the cell.

The chemicals bioavailability factor is described as the rate of mass transfer relative to the intrinsic activity of the microbial cells and controlled by several physical and chemical parameters, such as sorption/desorption, diffusion and dissolution (Cuypers et al., 2001). A low bioavailability is caused by a slow mass transfer to microorganisms and means longer time to get the bioremediation aims.

The most important chemical parameter for this phenomenon is the desorption, described usually in terms of desorption kinetics. In fact, usually, after a first period of fast desorption, a long period of slow desorption occurs, affecting also the PAHs removal, that will be fast in the first phase and then it will become always slower, due to the reduced PAHs desorption.

It is easy to understand that bioavailability is an important aspect of bioremediation and unfortunately, PAHs removal during bioremediation is often incomplete due to the low value of this factor, generating concentrations too high to satisfy the standards for clean soil. For this reason in the last years, lots of method to increase the pollutants bioavailability have been developed such as solvent extraction and supercritical CO_2 extraction, that are the oldest ones, till the cyclodextrin extraction and surfactant extraction (Cuypers et al., 2001).

- **Ageing**

The ageing is a natural process that takes place in soils when contaminated compounds are present for long time, decreasing the bioavailability of contaminants. According to Alexander (2000), as they age in the soil, contaminants become less available for the microorganisms activities, and so for bioremediation process. But to this low bioavailability is also connected a decrease of their toxic effects. It is really important in case of ancient polluted sites bioremediation to consider the ageing phenomenon, otherwise an overestimation of the environmental problems caused by the pollutants can occur. Concerning toxicological significance, ageing is an important aspect since the acute and chronic toxicity of pollutants declines as they remain stored in the soil.

The mechanisms concern the slow movement of pollutants into small sites of soil matrix that are not readily accessible by microorganisms. According to Xing (2000), humic acids are the responsible compounds for the sorption of hydrophobic molecules in soils, since they constitute mostly the organic matter present in soils. However it is suggested that the organic soil matter can not be the only soil property that is important; for example also the presence of micro-pores and voids should be really important.

It is also important to remember that the percentage of compound that is not available in the soil due to the ageing differs also among different soils and different pollutants, suggesting a characterization of each single case before proceeding with the bioremediation processes.

- **Co-metabolism**

Co-metabolism is defined as a non specific enzymatic reaction, with the secondary substrate competing with the primary one, which is usually structurally similar, for the enzymes active sites. In addition the transformation of the secondary substrate, also called co-substrate, is without any energetic benefit for the degrading bacteria, which use only the primary substrate to grow. The co-substrate is not assimilated by the bacteria, but the products could be available for other microorganisms species, in case of mixed cultures.

As showed by Fritsche and Hofrichter (2000), the substrate needed by the microorganisms to grow is easily degradable, usually aliphatic compounds as methane or ethane. During this degradation, unspecific enzymes are produced to degrade the first substrate, but they can also attack other compounds, such as aromatic or halogenated compounds, favouring their biodegradation. In another paper, Ambrosoli et al. (2005) suggest a co-metabolic enhancement of PAHs degradation, using glucose or acetate as co-substrate. They connect the higher degradation rate to an increase of microbial population that leads to improve the biodegradation of xenobitic compounds. Also Yuan and Chang (2007) report that the biodegradation of two and three rings PAHs can be enhanced with addition of pyruvate, lactate and acetate, even though in some cases an inhibition of microorganisms can occur.

1.3.2 Aerobic biodegradation

The most rapid and complete degradation of the majority of pollutants is brought under aerobic conditions. As said before, oxygen is the best electron acceptors that may take part in this process, since it provides the higher energy level to the microorganisms, yielding a higher growth rate. This higher energy supply has reference to a higher production of ATP moles through the aerobic metabolic pathway.

Usually, in the aerobic metabolism the initial step is always a enzymes catalyzed reaction like a mono-oxygenases or a di-oxygenases, which lead to hydroxylated compounds and to the ring cleavage and subsequently to the formation of CO_2 and water (Wentzel et al., 2007). In particular in case of polycyclic aromatic compounds, the di-oxygenase enzyme operates to form a *cis*-hydrodiol, which is transformed to carbon dioxide through other enzymes-catalyzed reactions, as dehydrogenases, isomerases and aldolases (Bamforth and Singleton, 2005). Oxygen is needed by the degrading microorganisms at two metabolic sites, at the starting attack of substrate and then at the end of the metabolic chain to form water.

There is a large variety of bacteria able to degrade aromatic compounds under aerobic conditions using di-oxygenase enzymes, as *Pseudomonas* strain, which seems to have the highest degrading potential (Fritsche and Hofrichter, 2000),

Sphingomonas strain, that showed the most extensive degradation of 4 and 5 rings PAHs (Mueller et al., 1997) and *Mycobacterium* strain (Bastiaens et al., 2000).

1.3.3 Anaerobic Biodegradation

In a typical aerobic system, the oxygen concentration is directly related to its solubility in water, that is around 7 ~ 8 mg/l (at room temperature), and usually this amount is enough to assure the oxygen supply to the bacteria, especially in the subsurface environments. However, in deep sediments, it could happen that the oxygen consumption rate is higher than the supply, due to the slow dissolved oxygen diffusion into sediments. This leads to the formation of anaerobic areas, forcing the bacteria, which are able to do it, to change their metabolism, slowing down the degradation process (Cerniglia and Heitkamp, 1989).

Indications that metabolism of aromatic structures is possible under anaerobic conditions in nature, were firstly provided by Tarwin and Buswell (1934, cited by Taylor et al., 1970). However, only since the late 1980s, an increasing number of novel microorganisms have been shown to utilize saturated and aromatic hydrocarbons as growth substrate under strictly anoxic conditions (Mihelcic et al., 1988; Kuhn et al., 1987). Microorganisms utilize nitrate, ferric ions and sulfate as electron acceptors for anaerobic respiration and the mechanisms of activation of hydrocarbons are completely different from those employed in aerobic metabolism. These other electron acceptors are more water-soluble and for this reason in case of enhanced biodegradation they never represent the limiting factor.

It is usual to find anaerobic conditions deeply in the ground, but it could also happen that aerobic environments develop anaerobic conditions due to the stimulation of natural degradation processes, that leads to the decrease of the molecular oxygen level (Heider and Fuchs, 1997). This oxygen is not replaced at the same rate of its depletion, creating anaerobic areas around the contaminating source (Bamforth and Singleton, 2005). However, anaerobic degradation is slower than the aerobic one due to a lower energy production during the bacteria metabolism, involving a low growth rate of microorganisms, and it degrades a smaller range of compounds (Wentzel et al., 2007).

In case of anaerobic metabolism is fundamental to replace all oxygen dependent steps by a new set of reactions and the formation of different intermediates, for breaking the aromaticity and cleaving the ring, that is reduced rather than oxidized. In addition, in absence of oxygen, redox potential plays a main role in determining the metabolic diversity of microbial population in soils, even though there is not a precise redox potential value for each condition. To obtain anaerobic reductive conditions the redox potential must display values close to or under 0mV. Nevertheless it is not easy to find in the literature concordant data about the correct redox potential range for each condition. In the paragraph 1.3.4.5 the characteristics of redox potential values and their influence on the bioremediation process will be properly discussed.

1.3.3.1 Nitrate reducing conditions

Under nitrate reducing conditions, the nitrate has the function of electron acceptor and is reduced till gaseous nitrogen. The sequence of intermediate products is NO_3 reduced to NO_2 and then to NO, N_2O and finally to N_2 that is subsequently released into the atmosphere; the all process is called denitrification and is similar to the one that happens in the water waste cycle (Paul and Clark, 1996).

The stoichiometry of the electron acceptor half reaction connected to the nitrate reducers degradation process, as reported by Rittman and Mc Carty (2001), is:

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \rightarrow \frac{1}{10}N_2 + \frac{3}{5}H_2O\,.$$

Rocken and Strand (2001) report the half reaction for the complete mineralization of naphthalene to CO_2 coupled to denitrification:

$$\frac{1}{48}C_{10}H_8 + \frac{5}{12}H_2O \rightarrow \frac{5}{24}CO_2 + H^+ + e^-$$

$$\frac{1}{5}NO_3^- + \frac{6}{5}H^+ + e^- \rightarrow \frac{1}{10}N_2 + \frac{3}{5}H_2O\,.$$

Adding the two equations gives an overall nitrate/PAH stoichiometry of 9.6 moles nitrate per mole of naphthalene.

In case phenanthrene is the carbon source, the stoichiometry is similar:

$$C_{14}H_{10} + 28H_2O \rightarrow 14CO_2 + 66H^+$$
$$66H^+ + 13.2HNO_3 \rightarrow 6.6N_2 + 39.6H_2O.$$

In literature there are several examples that demonstrate the degradation operated by soil microorganisms towards polycyclic aromatic hydrocarbons. For instance, Mihelcic and Luthy (1988) showed the biodegradation of naphthalene and acenaphthene under denitrifying condition, in limiting or excess nitrate conditions. They observed, in soils not previously exposed to PAHs, an acclimatization period between 12 and 24 days for naphthalene and 12 and 36 days for acenaphthene, even though the mineralization of soil organic carbon has started immediately. Under the nitrate-excess condition a degradation of both PAHs to non detectable levels was shown in less than 9 weeks. The acclimatization time is attributed to the time required to a small population of PAH degrading bacteria to gain a sufficient dimension to show a detectable PAHs degradation.

Rockne and Strand (2001) show a direct relation to the decrease of naphthalene and phenanthrene, the decrease of nitrate and to the production of N_2O, demonstrating the role of alternative electron acceptor plays by the nitrate and that a lack of this compound is a limiting factor for PAHs biodegradation. In this case, starting from a concentration of ~ 5 mg/l for naphthalene and ~ 600 μg/l for phenanthrene, an almost complete removal of phenanthrene is showed, whereas a partial mineralization of naphthalene, in about 80 days.

In addition, Uribe-Jongbloed and Bishop (2007) describe a higher degradation of 4 PAHs (naphthalene, phenanthrene, pyrene and benzo(a)pyrene) under high-share conditions instead of no share ones. Naphthalene, in both cases, showed a different behaviour compared to the other PAHs, displaying a higher degradation (in case of high-share conditions, the all naphthalene amount is deplete to non detectable level in 85 days). The other compounds degradation is at the end of the experiment around the 30 – 40%. This difference is explained to be directly connected to the significantly higher water solubility of this organic compound, which allows a major bioavailability for microorganisms.

Finally, Yuan and Chang (2006) report that biodegradation under nitrate-reducing condition is inhibited by the addition of lactate, pyruvate and acetate, used to favour the co-metabolism that usually enhance the biodegradation.

1.3.3.2 Sulphate reducing conditions

Under sulphate-reducing conditions, sulphate plays the role of alternative electron acceptor. Sulphate reducers, also known as sulphidogens, are described obliged anaerobes, for which the proper conditions requires a redox potential about -200 mV. Due to the rapid decrease of oxygen, nitrate and ferric ions in marine sediments, and the abundance of sulphate in seawater, sulphate reduction is often describe as the main degradation process in the marine environment. Sulphate bacteria as an organisms group degrade a broad range of compounds, although the degradation capacity of individual species seems more restricted then within denitrifying bacteria (Heider and Fuchs, 1997).

The stoichiometric electron acceptor half reaction connected to sulphidogens degradation process, as reported by Rittman and Mc Carty (2001), is:

$$\frac{1}{8}SO_4^{2-} + \frac{19}{16}H^+ + e^- \rightarrow \frac{1}{16}H_2S + \frac{1}{16}HS^- + \frac{1}{2}H_2O\,.$$

In case phenanhrene is the carbon source, according to the presented stoichiometry, the reaction is:

$$C_{14}H_{10} + 28H_2O \rightarrow 14CO_2 + 66H^+$$
$$66H^+ + 8.25H_2SO_4 \rightarrow 8.25H_2S + 33H_2O.$$

Teske et al. (2003) demonstrate that the intermediate usually generated by the sulphate reduction is the sulphite (SO_3^{2-}). The reaction is catalyzed by the key enzyme adenosine-5-phosphosulphate reductase as described by Friedrich (2002). Subsequently the sulphate would have been transformed in sulphide, defined here as the sum of H_2S, HS^- and S^{2-} (Schrot et al., 2001), through a catalyzed reaction by a sulphite reductase enzyme.

Concerning the sulphate biodegradation pathways, carboxylation is often suggested as the first catalyzed reaction, that leads to formation of carboxylic acids, then to the ring cleavage and with further step to the complete mineralization of the polycyclic aromatic hydrocarbons (Zhang and Young, 1997 and Meckenstock et al., 2000). However, another author, Bedessem et al. (1997) has proposed hydroxylation as the initial step reaction under the same conditions. For the other anaerobic conditions CoA ligase, oxidoreductases and decarboxylase are proposed as the first step for the metabolic pathway (Heider and Fuchs, 1997).

Studying the degradation mechanisms, Lovely et al. (1995) have been the first that demonstrate the relation between the aromatic compounds degradation and the decrease of sulphate in sulphate-reducing environments, describing this compound as the main alternative electron acceptor in the experiment. This thesis has been supported by the fact that when the sulphate was depleted, the benzene degradation stopped, but if the sulphate is added again, the biodegradation resumed.

Coates et al. (1996), studying the PAHs anaerobic degradation of marine sediments, found out that PAHs degradation is directly linked to the degree of PAHs contamination in sediments. For this reason they asserted that under sulphate-reducing conditions, long term exposure to PAHs may be necessary before a significant PAH-degrading community can be established. In a further publication (Coates et al., 1997) they have supported this thesis, suggesting a pre-exposure of microorganisms to high PAHs concentrations to obtain the development of a microbial community that is able to degrade rapidly PAHs under anaerobic conditions. In addition, they found out that molybdate is a specific inhibitor of sulphate reduction, providing a method to detect that sulphate-reduction is really happening.

Further studies, basing on Lovely thesis, focused on the long term (1 year) monitoring of marine harbor sediments, where strict anaerobic conditions have been found, suggesting good conditions for sulphate-reducing bacteria (Rothermich et al., 2002). They provided also sulphate to the sediments, trying the biostimulation method to enhance PAHs biodegradation. They realized that generally smaller PAHs are degraded more quickly than the larger ones, since after 105 days of monitoring, also for the stimulated sediments, no degradation was detected for high molecular weigh PAHs, that are the 4 and 5 rings aromatic compounds (fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene and benzo(k)fluoranthene). The only exception has been the benzo(a)pyrene, which presented a degradation of 8% of the initial amount after 105 days. However at the end of the experiment (day 338) a degradation, even though not so big, has been detected also for this compounds, demonstrating for the first time that sulphate bacteria are able to degrade also HMW PAHs. Concerning the LMW PAHs, really good degradation has been obtained already after 105 days, with the only exception of naphthalene, confirming that

natural sulphate-reducing bacteria may remove significant quantities of the PAHs contamination under anoxic conditions.

Finally Tsai et al. (2008) carried out studies about the sulphate bacteria enrichment to stimulate the biodegradation of polycyclic aromatic compounds. In particular they focused on fluorene and phenanthrene, suggesting also the simultaneous presence of both compounds, since the biodegradation is favoured via a co-metabolic process. The reported concentration are based on PAHs analysis in the aqueous solution and on PAHs absorbed by the biomass. They obtained a degradation of almost 75% of fluorene and only 61% of phenanthrene in 21 days after reaching proper conditions for bacteria, even though a loss equal to 15% of fluorene and 17% of phenanthrene is detected in the abiotic control. Both degradation rates showed two phases: an initial phase with rapid degradation (from day 0 to day 9) and a further phase where degradation becomes slower and longer (from day 10 to day 21).

1.3.4 Factors affecting the biodegradation process

Several factors are reported in literature affecting the biodegradation processes. In fact chemical parameters as pH, redox potential, temperature and electron acceptors concentration can play an important role and, if they become a limiting factor, they are able also to inhibit growth of the pollutant degrading microorganisms (Bamhfort and Singleton, 2005). The main environmental factors that could affect the feasibility of bioremediation are summarized in the following sections.

1.3.4.1 Temperature

The temperature has considerable effects on the ability of microorganisms to degrade pollutants. Due to this reason, it is appropriate to say that, concerning the *in situ* bioremediation, not every period of the year is suitable.

Usually for a class of microorganisms it is possible to delineate a bell-shaped trend, identifying a maximum growth value in a small range of temperature. Basing on the different temperature affinity, related to the temperature range in which the highest growth is occurred, there are different microorganism classes:

Class	Temperature Range (°C)
Psychrophiles	Lower than 15
Mesophiles	20 – 40
Termophiles	45 – 65
Hypertermophiles	Higher than 80

Table 1.4 – Temperature range for different microorganisms classes

Moreover, as describe by Barkay et al. (1999), the PAHs solubility is a function of temperature and particularly it increases with an increase of temperature, enhancing the bioavailability of contaminant compounds. But at the same time, in case of aerobic biodegradation, the oxygen concentration decreases with a temperature increase, reducing the microbial activity since the electron acceptor becomes a limiting agent.

Bioremediation, generally, can occur over a wide temperature range, however most studies tend to focus on mesophilic conditions, rather than with low or high temperatures. Nevertheless, some scientific publications report that microorganisms are able to degrade polycyclic aromatic hydrocarbons also at extreme temperature. Examples are present in literature of biodegradation of naphthalene and phenanthrene in cold seawater with a temperature around 0°C (Siron et al., 1995) and also fungi that are able to degrade more than 90% of contaminating PAHs at temperature higher than 75°C (Lau et al., 2003).

1.3.4.2 pH

In the same way of temperature, also pH has the ability of influence the degradation process, operating on the microorganisms growth rate. Inhibition can occur with too elevated or too low pH values, so it is common practice to adjust the pH to neutrality, as reported by Margesin and Schinner (2001).

In case of sulphate-reducing conditions common values for degradation are around 7 – 7,5. Tsai et al. (2008) report a pH value of 7.3 – 7.4, obtaining a degradation of 75% for fluorene and 61% of phenanthrene. Similar conditions were detected by Yuan and Chang (2006) both for nitrate and sulphate-reducing conditions, with a pH range variable between 6,8 and 7,2. Another author, Zaidi and Imam (1999) reported

that varying the pH from the neutrality to 6.0 or 8.0 didn't have big effects on the phenanthrene, but a variation of pH to 10.0 had more serious effects since the degradation was markedly reduced.

Finally PAHs degradation has been recorded in a acidic soil, with a pH equal to 2, contaminated by coal spoil. In this case the bacteria have been able to produce a 50% degradation percentage for naphthalene, 20% for anthracene and 10% for phenanthrene (Stapleton et al.,1998).

1.3.4.3 Nutrients

During the a soil bioremediation process is important to check and supply, in case of lack, nutrients to support the biomass growth and energy generation. Nutrients, being elements comprising the physical structure of cells, are needed in proportion to the net production of biomass. The need of nutrients is proportional to the degradable carbon source.

The nutrients required by microorganisms are nitrogen, phosphorus and potassium. In contaminated sites, usually the available carbon levels are high and nutrients as N and P can be quickly depleted due to the microbial metabolism. Monitoring the concentration of this compounds is crucial for a successful bioremediation process (Alexander M., 1999). For this reason in literature the C:N:P ratio has been studied and it varies between 100:15:3 to 120:10:1 (Bamforth and Singleton, 2005). Other authors (Alexander, 1999 and Rittman and Mc Carty, 2001) stated that the optimal ratio is approximately 100:10:1. However it must be remembered that a high nutrients load can inhibit microbial degradation.

Many bacteria also require low concentrations of one or more amino acids, B vitamin, fat-soluble vitamins or other organic compounds. The need of these compounds is due to the microorganisms growth (Alexander, 1999).

1.3.4.4 Electron acceptors

The natural availability of electron acceptors has a fundamental importance for the biodegradation process. If a lack of electron acceptors occurs, the whole bioremediation process is stopped since it is not possible to transfer electrons from the contaminant compounds. The electron acceptors are consumed in proportion to the electron donors. Obviously the presence of the electron acceptors is directly

connected to the water solubility and the transfer rate of these compounds. In the following table the water solubility of the main electron acceptors acting in these processes are reported.

Electron Acceptor	Water solubility (mg/l)
Oxygen	7 ~ 8 (at 20°C)
Nitrate	880.000 (at 20°C)
Sulphate	440.000 (at 20°C)

Nitrate value refers to sodium nitrate solubility (Perry and Green, 2007)

Sulphate value refers to sodium sulphate solubility (Perry and Green, 2007)

Table 1.5 – Water solubility of different electron acceptors

For nitrate and sulphate the water solubility does not represent a problem, since the value is high and it is enough that the depletion rate is equal to the supply one. However problem can be generated by the end products: in case of nitrate, the end product, nitrogen is poorly water soluble and bubbles could create in the soil, excluding water from pore spaces; in case of sulphate, the intermediate compound, the sulphide could be toxic for microorganisms. Genthner et al. (1997) reported that the good stoichiometric concentration for these chemicals is 15 – 20 mM.

On the other side, for oxygen the solubility value is low and it can represent a limiting factor, especially in case of deep environments. In addition the aerobic degradation is more energetic and so quickly than the anaerobic one, meaning that in case of high contamination, oxygen could represent a limiting factor. For these reason several methods have been developed to increase the oxygen concentration in water, such as the air sparging or the addition of oxygen releasing compounds, as hydrogen peroxide and ozone (van Cauwenberge and Roote, 1998).

1.3.4.5 Oxidation reduction potential

In order for energy to be released from an oxidation reduction reaction, an overall negatives must exist, so that the reaction is thermodynamically favourable. In case of anaerobic biodegradation the Gibbs's energy is positive, meaning that microorganisms need to use energy, previously produced by them, to degrade

contaminants. For this reason the anaerobic process is told to be endergonic, producing less energy than the aerobic one. The oxidation reduction potential (ORP) and the Gibbs's free energy are connected by the equation:

$$E_0 = -\frac{\Delta G}{nF}$$

where:

E_0 = standard redox potential,

ΔG = Gibbs free energy,

n = number electrons moles for moles of products,

F = Faraday's constant = 96485 C/mol.

At a particular redox potential a single electron acceptors will be favoured by these thermodynamic considerations. If oxygen is present, as said, it is the main electron acceptor because it has the highest energy production: typically electron acceptors are utilized by the bacteria in order of their energy yield, from highest to lowest. This means that after oxygen there is nitrate, iron reduction, sulphate-reducers and methanogensis. However, the literature data are not agreeing, especially for anaerobic conditions.

Typical ORP values for aerobic conditions are around 800 - 850mV (Karthikeyan and Bhandari, 2001 and Rockne and Reddy, 2003). In case of anaerobic degradation, different information are present from the literature (see table 1.6).

Nitrate ORP (mV)	Sulphate ORP (mV)	Authors
~700	~(-200)	Karthikeyan and Bhandari (2001)
~800	~(-80)	Rockne and Reddy (2003)
(-100) ~(-260)	(-100)~(-380)	Yuan and Chang (2006)
	(-210)~(-350)	Tsai et al. (2008)
~300		Ambrosoli et al. (2005)

Table 1.6 – ORP for different anaerobic conditions

It is clear that ORP negatives values are needed in case of sulphate-reducers degradation and this is due to the fact that these microorganisms are strictly anaerobic and can not survive in presence of oxygen. In case of nitrate-reducers the

values are different due to the fact that these bacteria are usually facultative aerobes and can survive also in presence of oxygen.

1.3.5 Metabolic pathways

To know the metabolic pathways of PAHs degradation is important to understand the final fate of contaminants, if they become more toxic or less dangerous for the environment. In addition it is also possible to predict the biodegradation simply checking and monitoring the intermediate metabolites production.

Common to both types of metabolism, anaerobic and aerobic, is a separation into peripheral and central pathways (Heider and Fuchs, 1997). The *peripheral pathways* convert the large variety of aromatic compounds in a few central aromatic intermediates. Usually the enzymes able to act in this transformation are coded by genes on plasmids, are not very specific for particular substrate and are induced just by the presence of the substrate. The central intermediates produced by the peripheral pathways are usually ready to be dearomatised through the *central pathways*, which differentiate basing on the metabolism and the degraded contaminants.

In both metabolisms the first goal is to remove the aromaticity and introduce hydroxyl functions. The favourite enzymes able to catalyzed this reaction are the mono-oxygenase, typical for linear hydrocarbons degradation, and di-oxygenase, typical for PAHs microbial degradation (Bamforth and Singleton, 2005). In case of anaerobic process oxygen is not available and the most reliable metabolism first step is the carboxilation of the aromatic ring (Zhang and Young, 1997, Zhang et al., 2000, Meckenstock et al. 2000). Nevertheless, other authors (Tsai et al. 2008) report that no carboxylic acids have been detected during the anaerobic degradation of phenanthrene and fluorene. Then, in the second part, some enzymes intervene supporting the ring cleavage and the further transformation in the last products of degradation process. The main target is to obtain the mineralization of the initial pollutant, but sometimes it is just possible to obtain the transformation in other compounds, that are not always less toxic and dangerous than the initial ones.

1.3.5.1 Anaerobic metabolic pathways

Anaerobic metabolic pathways are not substantially present in literature, and only in the last 10 years studies were carried out to investigate the intermediates of anaerobic processes. In addition most of these studies are focused on the metabolic pathways of naphthalene, since it is one of the easiest PAHs to degrade. Also the phenanthrene metabolic pathway has been investigated in the last years, but further studies are recommended.

Two authors, Zhang et al. (2000) and Young and Phelps (2005), show the same metabolic pathway for naphthalene and 2-metylnaphthalene biodegradation. The first described reaction is a carboxylation of the aromatic compound, which leads to the formation of 2-naphthoic acid. Then, through an hydrogenation, 2-naphthoic acid is dearomatised to 5,6,7,8-tetrahydro-2-naphthoic acid. A further hydrogenation produces a fully saturated decalin-2-naphthoic acid, which then get further metabolized to carbon dioxide (Figure 1.6).

Young and Phelps (2005) identified also 2-naphthoic acid as a possible naphthalene degradation biomarkers, since it is not usually present in soil and it is specific for the interested process.

Another author, Annweiler et al. (2002), reveals that probably decahydro-2-naphthoic is a dead end metabolite and suggests the formation, after the 5,6,7,8-tetrahydro-2-naphthoic acid, through an hydration of hydroxydecahydro-2-naphthoic acid, then oxidized to oxodecahydro-2-naphthoic acid. A thiolytic ring cleavage and a β-oxidation bring to the formation of carboxycyclohexylacetic acid. However they have not been able to describe the further steps to the complete mineralization.

Zhang and Young (1997) suggest a similar pathway for phenanthrene, even though they were not able to confirm the exact junction position of carbon dioxide for the formation of the carboxylic acid.

Tsai et al. (2008) present an alternative metabolic pathway, because they did not detected carboxylic acids during the monitoring degradation. They propose the degradation through the formation of phenol, that is subsequently transformed in acetic acid.

The initial sequence which leads to the formation of p-Cresol is unclear for the authors. Then it is suggested a sequence of hydroxylations, with the formation of p-Hydroxybenzyl alcohol, p-Hydroxybenzyl aldehyde and p-Hydroxybenzoate. Then, through a decarboxylation, phenol is obtained. Finally the phenol is bio-transformed in acetic acid, which is easily metabolized by microorganisms (Heavy and Young, 1978, Boyd et al., 1983) and mineralized to water and carbon dioxide.

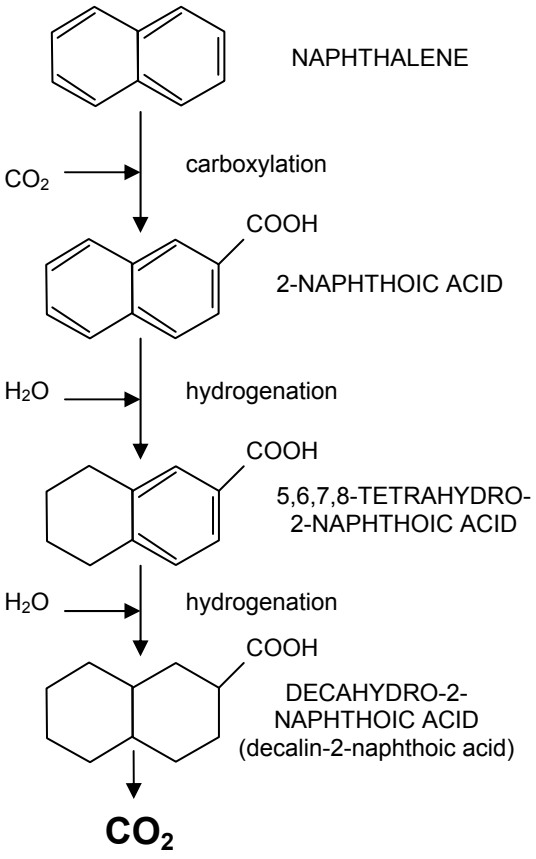


Figure 1.6 – Naphthalene anaerobic metabolic pathway, (Zhang et al. 2000, Young and Phelps, 2005)

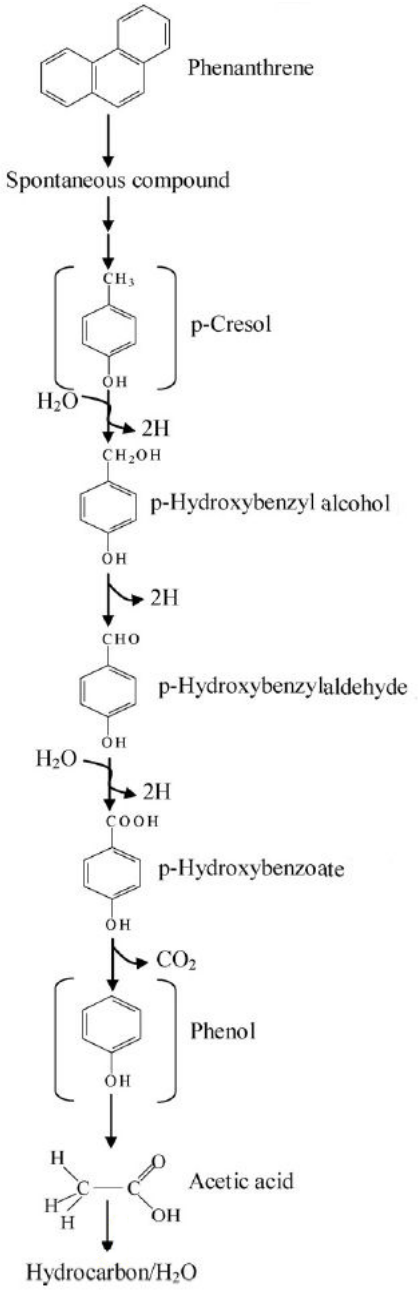


Figure 1.7 – Phenanthrene anaerobic metabolic pathway, (Tsai et al., 2008)

1.3.5.2 Aerobic metabolic pathways

The PAHs aerobic metabolic pathways have been more studied in the last decades and also for high molecular weigh PAHs these pathways are known. In this dissertation only the naphthalene metabolic pathway will be present, to allow a comparison with the anaerobic one.

As said, the first enzymatic catalyzed reaction is the oxidation of the aromatic ring, with the formation of cis-1,2-dihydroxy-1,2-dihydronaphthalene, which subsequently is dehydrogenated to 1,2-dihydroxynaphthalene.

Then through two reactions, one catalyzed by an enzyme isomerase and the other by the enzyme aldolase, the salicylaldheide is obtained, as described by Grund et al. (1992). After that, with other two reactions, catechol is obtained. Hence the intermediate obtained is easy for microorganisms to mineralized through the Krebs Cycle. For the other PAHs the metabolic process is similar, requiring always a dioxygenase as first metabolic step.

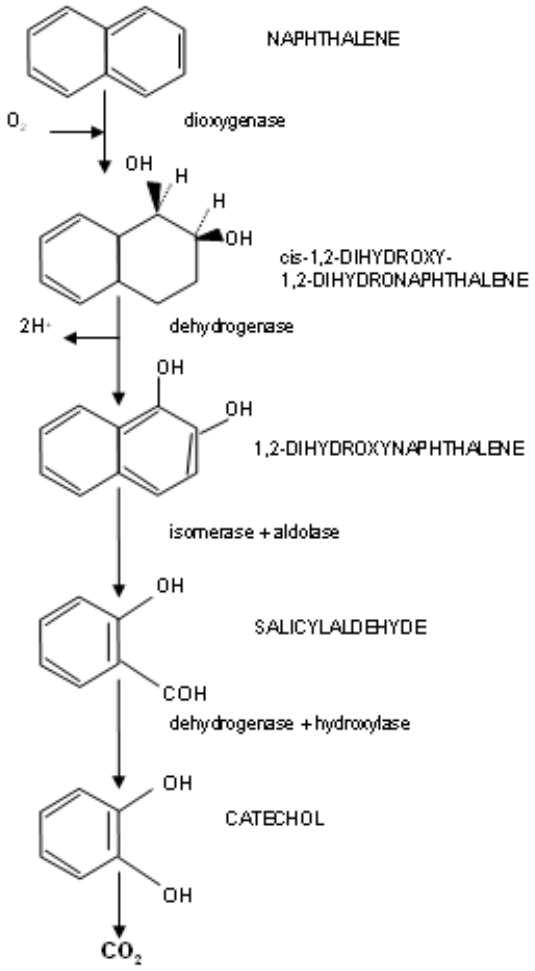


Figure 1.8 – Naphthalene aerobic metabolic pathway, (Grund et al., 1992)

Chapter 2
Aim of the thesis

The aim of this work is to characterise the conditions favouring the anaerobic biodegradation of organic pollutants. Special focus will be given towards the assessment of the efficiency of various AEAs, the characterisation of degradation performance and rates under different redox conditions as well as toxicity monitoring. Consequently, a series of investigations to prove applicability and predict remediation performance will be performed. Various preliminary tests include degradation experiments in shaking flasks.

In particular polycyclic aromatic hydrocarbons biodegradation will be studied. Two different anaerobic conditions will be investigated to assess the efficiency of different alternative electron acceptors: nitrate and sulphate reducing conditions. Additionally an aerobic experiment will be performed with the same material, to compare the different condition, assessing the different degradation rates.

In order to simulate material present in the vadose zone all experiments will be performed with sand material artificially spiked with five different PAHs and additionally suspended in mineral medium containing nutrients and electron acceptor.

The attention will be concentrated not only on the biodegradation process itself, but also on the chemical and physical parameters that allow the development of optimal conditions for the bioremediation. A fixed parameter will be the temperature, which will be set on the room temperature (20 – 22°C). In addition, to get hydrodynamic conditions, samples will be shaken constantly at 180 rpm.

This work is a part of an Austrian national project (NUTZRAUM) and it has been performed at the Interuniversitäres Departement für Agrarbiotechologie (IFA Tulln), a department of Universität für Bodenkultur Wien, in the research group Contaminated Land Management, for a length time of 6 months, from August 2008 to January 2009.

Chapter 3

Materials and methods

Two different experiments have been carried out using a sand under aerobic and anaerobic conditions. In particular, an unpolluted sea sand sample, subsequently contaminated with 5 different PAHs has been used. Afterwards, the sand has been suspended into a mineral medium, containing dissolved chemicals useful for the growth of microorganisms and nitrate or sulphate as alternative electron acceptors in case of anaerobic conditions and oxygen as electron acceptors for aerobic degradation conditions. The degradation experiments were conducted as laboratory-scale tests in shaking flasks under constant shaking and uniform environmental conditions.

3.1 Degradation experiments outline

3.1.1 Anaerobic experiment

Concerning the anaerobic experiment structure, 3 different parallel testing lines have been prepared:

1. *nitrate reducing conditions* testing line (PAH NO_3), in which the electron acceptor for the redox reaction is the nitrate (NO_3^-);
2. *sulphate reducing conditions* testing line (PAH SO_4) in which the electron acceptor for the redox reaction is the sulphate (SO_4^{2-})
3. *abiotic control* testing line (PAH AC) which represent the condition of complete absence of microorganisms and it is useful to check the degradation rate that is assigned just to a physical loss.

A total of 6 sampling days have been predeterminate to monitor anaerobic PAH degradation. For each sampling day 4 samples has been created for each testing line. The first 3 samples are needed to investigate the concentrations of PAHs in the solid

and liquid phases; particularly the number 3 is expressly decided to supply a statistical validity to the obtained results. The fourth sample is utilized to monitor the change in chemical and physical parameters typical for anaerobic process, displayed in the following list:

ORP (Oxidation Reduction Potential)
pH
σ (conductivity)
O_2 (oxygen concentration)
T (temperature)
DOC (dissolved organic carbon)
NO_3^- (nitrate ion)
SO_4^{2-} (sulphate ion)
NO_2^- (nitrite ion)
SO_3^{2-} (sulphite ion)
S^{2-} (sulphide ion)
NH_4^+ (ammonium ion)
PO_4^{3-} (phosphate ion)

The temporal structure of the sampling days is the following: day 1, day 34, day 55, day 83, day 111, day 130. Additional samples, one for each line, have been created to monitor the conditions during the first weeks, reaching proper conditions for anaerobic bacteria. In the following table the anaerobic experiment lay-out is displayed.

Day	1	34	55	83	111	130
Nitrate-reducing condition	x	x	x	x	x	x
Sulphate-reducing condition	x	x	x	x	x	x
Abiotic control	x		x		x	x

Table 3.1 – Anaerobic experiment lay-out

3.1.2 Aerobic experiment

Regarding to the aerobic conditions experiment, the structure turns out to be the same of the anaerobic experiment, even though only two different testing lines are:

1. *aerobic conditions* testing line (O_2 PAH), in which the oxygen is the electron acceptor in the oxidation-reduction reaction;
2. *abiotic control* conditions (O_2 PAH AC) with the complete absence of any living microorganism.

Compared to the anaerobic case, there are some differences:

→ for the abiotic control testing line, no sample is scheduled for the monitoring of process parameters, i.e. only three samples are prepared for each sampling day to analyze the PAHs concentration;

→ for the aerobic conditions line, the sample needed to check the process parameters is scheduled only in the sampling days in which also the abiotic control is present.

Concerning that theoretically the aerobic degradation is faster than the anaerobic one since the aerobic respiration is more esoergonic (Section 1.3.2), following sampling programme is scheduled:

Day	1	7	14	21	55	83
Aerobic condition	x	x	x	x	x	x
Abiotic control	x			x		x

Table 3.2 – Aerobic experiment lay-out

3.2 Samples preparation

Once the experiment is set, the samples preparation is performed, which includes the PAHs solution preparation, the sand spiking step and the mineral medium solution preparation. At the end of this procedure, the samples are placed in a shaker which allows to create dynamic conditions inside the samples, with a rotatory motion equal to 180 rpm/min.

3.2.1 Spiking

The material chosen for the experiments is a sea sand, coming from *Aldrich Industries*, SiO_2, +70 mesh, white quarz, suitable for chromatography.

3.2.1.1 Dry substance determination

The dry substance (or dry matter) determination is a fundamental step to fix the correct amount of contaminated sand needed in each sample's flask. Determination of the dry substance allows to get homogeneous conditions, avoiding the influence of water content, naturally present in sand, on results.

Approximately 5 g of sand are weighed into a small aluminium box, previously weighed to obtain the tare and subsequently dried in a drying chamber at 105°C (temperature that allows the water to evaporate, without influencing the organic matter amount). Afterwards, the dried sand is cooled down in a vacuum desiccator.

Samples for the determination of the dry substance are measured in triplicate, to assure a statistical validity of results. At this point it is possible to calculate the dry matter value as following:

$$DM = \frac{(W_1) - (T)}{(W_2)} \cdot 100$$

where:

DM = dry matter (%)

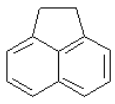
W_1 = dry sand weigh, including also the aluminium box (g);

W_2 = initial sand weigh (g);

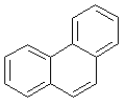
T = tare, weigh of aluminium box (g).

3.2.1.2 PAHs solution

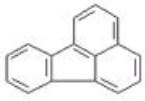
A solution of 5 main PAHs, frequently found in contaminated soils, has been prepared to contaminate the sand. The hydrocarbons chosen are:



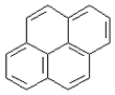
Acenaphtene



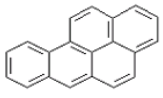
Phenanthrene



Fluoranthene



Pyrene



Benzo(*a*)pyrene

In this way, the fate of different polycyclic aromatic compounds is investigated, considering the different behaviour of each compound that is directly linked to the different chemical properties, elucidated in paragraph 1.2.

To choose the concentration of PAHs in the solution, data from real soils previous analysis were used. Referring to 1 kg of sand, the selected concentrations were:

PAH	Concentration (mg PAHs/kg sand)
Acenaphthene	38.52
Phenanthrene	191.17
Fluoranthene	1088.05
Pyrene	628.67
Benzo(a)pyrene	53.59
Total	2000.00

Table 3.3 – Target spiking PAHs concentration

However it must be considered that during the spiking step some losses are expected, due to the compounds volatilization. Concerning previous spiking studies performed at IFA Tulln, the following recovery percentages were taken into account during preparation of PAH solution:

PAH	Recovery Percentage
Acenaphthene	23.78
Phenanthrene	85.38
Fluoranthene	94.37
Pyrene	96.81
Benzo(a)pyrene	95.24

Table 3.4 – PAHs Recovery percentage

According expected loss during spiking procedure, the following concentrations of PAH in spiking solution have been calculated, prepared and later utilized to spike:

PAH	Concentration (g/l)
Acenaphthene	2.408
Phenanthrene	3.374
Fluoranthene	17.362
Pyrene	9.722
Benzo(a)pyrene	0.846

Table 3.5 – PAHs concentrations in the solution utilized to spike the sand

The solvent used to dissolve the toxic and carcinogenic chemicals is the dichloromethane (CH_2Cl_2), due to its high capacity to dissolve lots of chemical compounds (Brinch et al., 2002).

3.2.1.3 Spiking procedure

During the spiking procedure is really important that the contaminant and the sand are well mixed, so that it is possible to obtain a good polluted sand homogeneity level.

According to the procedure presented by Brinch et al. (2002) the PAHs solution is added to the sand sample and mixed for 5 minutes with a clean metal spoon. The mixing procedure is repeated after 1 hour, trying to support the solvent evaporation. Finally the samples are left for about 16 hours under the aspiration hood, to obtain the complete solvent evaporation.

In particular several beakers have been prepared, in which 20 ml of the prepared PAHs solution have been added to 300g of unpolluted sand. After complete solvent evaporation spiked sand was unified and well mixed to obtain homogenous material.

The spiking procedure has been the same for both anaerobic and aerobic experiment.

To determine PAHs content spiked material has been extracted with ethylacetate using soxhterm extraction procedure and analysed with the high performance liquid chromatography (HPLC).

3.2.2 Mineral medium

The mineral medium is the solution which commonly contains the substances necessary for the microorganisms growth, such as mineral salts and vitamins. In case of anaerobic experiment mineral medium additionally contains an alternative electron acceptors (AEA) required for the PAH biodegradation in absence of oxygen. Therefore, the mineral medium composition is different for each experiment and it depends also on the kind of microorganisms species present. In case of "abiotic control" testing line an appropriate chemical is needed to kill every living beings inside the solution.

The criterion used to calculate the mineral medium chemicals concentration, concerning the substances necessary for the microorganisms growth, is the same for both experiments: the ratio between carbon (C), nitrogen (N) and Phosphorus (P) is fixed to $C : N : P = 150 : 10 : 1$. Since no organic carbon is present inside the sand, for further calculations, the carbon concentration is chosen to be equal to the PAHs concentration in the sand.

3.2.2.1 Nitrate reducing conditions

In the case of anaerobic conditions with microorganisms nitrate-reducers, nitrogen is needed in the form of nitrate ions (NO_3^-) both for the microorganisms growth and as alternative electron acceptors and is added as sodium nitrate ($NaNO_3$).

Concentration of sodium nitrate needed for microorganisms growth is calculated according to ratio $C : N : P = 150 : 10 : 1$. Amount of nitrate serve as AEA is assessed due to stoichiometricaly calculated carbon/nitrogen ratio ($C : N = 1 : 0{,}94$). Further an excess of nitrate equal to 1/3 of the stoichiometric amount was added.

On the contrary of nitrogen, phosphorous is added in form of two phosphate salts (potassium dihydrogen phosphate, KH_2PO_4 and sodium hydrogen phosphate, Na_2HPO_4).

Finally, two solutions, necessary for the microorganisms growth, must be added. These two solution, previously produced at IFA Tulln, are the *trace elements* solution and the *vitamins* solution. In the following tables their composition is reported.

Chemical	Concentration (mg/l; ml/l)
H_3BO_3	3
$CaCl_2$	531
$CoCl_2$	3.9
$CuSO_4 * 5 H_2O$	17.3
$FeSO_4 * 7 H_2O$	206
KCI	101
$MnSO_4 * 1 H_2O$	14
$Na_2MoO_4 *2 H_2O$	5.3
Na_2SeO_4	0.6
$NiCl_2 * 6 H_2O$	4.6
$ZnSO_4 * 7 H_2O$	20.5
conc. H_2SO_4	1

Table 3.6 – Composition of trace elements solution

Chemical	Concentration (mg/l)
P-amino-benzoic Acid	5
Biotin	2
Folic Acid	2
Tiotic Acid	5
Nicotinic Acid	5
Pantotenic Acid	5
Piridossin HCl	10
Riboflavin (B2)	5
Thiamin	5
Cianocobalamin (B12)	0.1

Table 3.7 – Composition of vitamins solution

According to literature 10ml of trace elements solution and 1ml of vitamins solution per litre of mineral medium have been added.

Here below the summary of chemicals and solution necessary to prepare the nitrate reducing conditions mineral medium is listed:

Chemicals & Solutions	Concentration (mg/l; ml/l)
$NaNO_{3,\ AEA+GROWTH}$	4623
KH_2PO_4	19
Na_2HPO_4	20
Trace elements solution	10
Vitamins solution	1

Table 3.8 – Composition of nitrate reducing conditions mineral medium

Then, to obtain oxygen lack conditions, the mineral medium is degassed by blowing argon into the solution inside a mobile glove box, previously deprived of oxygen. The O_2 concentration is monitored with an electronic electrode and the degassing starts when the oxygen concentration under the mobile glove box is less than 0,10 mg/l.

Moreover, aerobic microorganisms from the IFA working bank have been added directly into the pre-degassed mineral medium in order to help in decreasing the possibly persistent oxygen in mineral medium and later in suspensions culture. The amount added is 1ml of bacteria solution in 1,5l of mineral medium solution

3.2.2.2 Sulphate reducing conditions

In the case of anaerobic conditions with microorganisms sulphate-reducers, sulphate is needed as electron acceptors and carbon, whereas nitrogen and phosphorous for the cellular growth. Then the process is the same of the previous one, using the same ratio for nutrients. Nevertheless, ammonium chloride has been used as nitrogen source for microorganisms growth instead of sodium nitrate.

About the alternative electron acceptors, the reasoning is the same of the case before, but the ratio changes. According to literature, the stoichiometric ratio between carbon and sulphur must be $C : S = 1 : 0{,}59$. As in case of nitrate reducers, also in this case it has been decided to work with an excess of AEAs, equal to 1/3 of the stoichiometric amount.

In addition, compared to the previous case, a reducing agent must be added, to reach the low redox potential required by these conditions (Section 1.3.4.5). The reducing

agent used has been sodium sulphide (Na_2S) and 0,001 mmol/ml of solution have been added.

Below we list the summary of chemicals and solution necessary to prepare the sulphate reducing conditions mineral medium:

Chemicals & Solutions	Concentration (mg/l; ml/l)
NH_4Cl	148 mg/l
KH_2PO_4	19 mg/l
Na_2HPO_4	20 mg/l
Na_2SO_4	4617 mg/l
Na_2S	78 mg/l
Trace elements solution	10 ml/l
Vitamins solution	1 ml/l

Table 3.9 – Composition of sulphate reducing conditions mineral medium

As the nitrate case, microorganisms were added to decrease possibly persistent oxygen after the mineral medium solution has been degassed with argon under a mobile glove box.

3.2.2.3 Abiotic control

As mentioned before, the abiotic control solution has the purpose of guaranteeing the total absence of microorganisms both in the anaerobic and aerobic experiment. The chemical utilized is the mercury-chloride ($HgCl_2$), that is a very toxic compound and it is largely used with this purpose due to its higher water solubility than other mercury compounds.

The concentration of $HgCl_2$ in the mineral medium was 0,1%. For both experiments, the anaerobic and the aerobic one, 1 litre of solution has been prepared, adding 1g of mercury-chloride and distilled water. Then, to obtain oxygen lack conditions, the solution is degassed by blowing argon into the solution inside a mobile glove box, previously deprived of oxygen.

3.2.2.4 Aerobic conditions

For aerobic experiment no alternative electron acceptors are needed, since the oxygen is naturally present in the mineral medium. The nutrients ratio $C:N:P=150:10:1$ is still respected and calculated basing on the initial carbon molar concentration. Nitrogen is added to the system as ammonium chloride, whereas phosphorous is provided splitting the amount between potassium dihydrogen phosphate (KH_2PO_4) and sodium hydrogen phosphate (Na_2HPO_4).

Finally trace elements solution and vitamins solution are added to the mineral medium in the same amount of the previous cases, that is 10ml of trace elements and 1 ml of vitamins per 1l of solution. Below the summary of chemicals and solutions is reported:

Chemicals & Solutions	Concentration (mg/l; ml/l)
NH_4Cl	134
KH_2PO_4	17
Na_2HPO_4	18
Trace elements solution	10
Vitamins solution	1

Table 3.10 – Composition of aerobic conditions mineral medium

3.2.3 Experiments Implementation

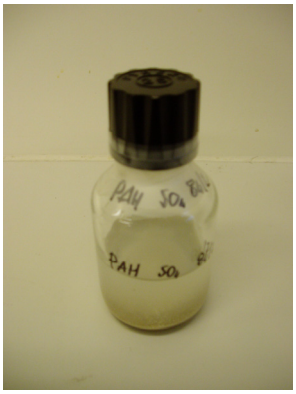
For each sample, 10g DM of contaminated sand and 40ml of mineral medium are put into a 100ml Pyrex flask. A difference between two experiments concerns the screw caps. In fact for the anaerobic testing lines a normal screw cap, with a Teflon seal, is placed and tightened directly under the mobile glove box, so that the sample is well sealed. Whereas for the aerobic samples the screw cap is only made of an aluminium sheet, on which holes are made to allow the sample aeration. For the abiotic control (aerobic experiment) a normal screw cap is used, but it is not strictly tightened, just fixed with a scotch tape.

In case of aerobic experiment, to help the optimal mixing, a spherical glass ball is put in every sample.

At the end the samples are placed on the shaker, which is at room temperature (20-22°C), and continuously mixed at 180 rpm. This shaker creates the dynamic conditions, that literature presents as better for the biodegradation than the stagnant conditions (Uribe-Jongbloed and Bishop, 2007). Below pictures of the samples and the shaker are reported.

Aerobic experiment sample (O2 PAH series)

Aerobic abiotic experiment sample (O2 PAH AC series)



Anaerobic experiment sample (PAH NO3/SO4/AC series)

Figure 3.1 – Different aspect of samples from aerobic and anaerobic testing lines

Figure 3.2 - Samples shaker

3.2.3.1 Microorganisms input

To introduce microorganisms inside the samples, different procedure have been applied, since to perform this step optimal environmental conditions are needed, principally concerning pH and redox potential.

For the aerobic case, the microorganisms have been put directly into the mineral medium since chemical parameters were already acceptable for microorganisms. For this reason 1,33ml of aerobic microorganisms solution, from the IFA master bank (Todorovic et al., 2001), have been added.

On the contrary, for the anaerobic experiment we had to wait for the achievement of suitable redox potential conditions. After 4 weeks from the begin of the experiment, 100µl of stock culture have been added into each sample. Afterwards 1ml of sludge, coming from a water waste treatment plant, particularly from the denitrification tank, has been added to the nitrate reducers testing line samples. The sludge characteristics are reported in the following table:

	ORP (mV)	pH	Lf (µS/cm)	O_2 (mg/L)	DOC (mg/L)
Sludge	-330	7,47	1,93	0,05	44,18

	SO_4 (mg/L)	NO_3 (mg/L)	NO_2 (mg/L)	SO_3 (mg/L)	NH_4 (mg/L)	PO_4 (mg/L)
Sludge	104,61	1,99	0,284	3,06	1,47	8,66

Table 3.11 – Denitrification tank sludge chemical parameters

3.3 Chemical parameters measurement

The control of chemical parameters, such as redox potential (ORP), pH, oxygen concentration (O_2), conductivity (σ), temperature (T), dissolved organic carbon (DOC), concentration of ions NH_4^+, PO_4^{2-}, NO_3^-, SO_4^{2-}, NO_2^-, SO_3^- and S^{2-}, are important to monitor PAH degradation.

Monitored parameters are different for each experiment (see table below).

Anaerobic Experiment	
ORP	SO_4^{-}
pH	NO_2^{2-}
σ	SO_3^{2-}
O_2	S^{2-}
T	NH_4^{+}
DOC	PO_4^{2-}
NO_3^{-}	

Aerobic Experiment	
pH	T
σ	DOC
O_2	

Table 3.12 – Chemical parameters monitored in both anaerobic and aerobic experiment

For the aerobic case, all 5 parameters are monitored at the days 1, 21 and 83. Whereas, for the anaerobic experiment following table reported parameters monitored at each sampling day.

Day	0	1	7, 14, 21	34	55, 83 ,111	130
Parameter						
PAHs (solid phase)	x	x		x	x	x
PAHs (liquid phase)		x		x	x	x
ORP	x	x	x	x	x	x
pH	x	x	x	x	x	x
σ	x	x		x	x	x
T	x	x	x	x	x	x
O_2		x	x	x	x	x
DOC		x		x	x	x
NH_4	x					
PO_4	x			x	x	x
NO_3	x			x	x	x
NO_2				x	x	x
SO_4	x			x	x	x
SO_3				x	x	x
S					x	
Toxicity		x		x		x

Table 3.13 - Chemical parameters monitored at each sampling day; anaerobic experiment

3.3.1 ORP, pH, O_2, σ and T

All these 5 parameters are monitored through an electrodes kit (*Hach Lange*, HQ 40d multi) connected with an electronic reader. The difference between the anaerobic testing line and the aerobic one is that for the first one every measurement must be performed in a mobile glove box (Captair Field Pyramid, 2200A) to avoid errors caused by the oxygen presence in the normal atmosphere.

Parameters are measured directly in the sample liquid phase, previously separated from the solid one. The separation is performed with a 40ml volumetric pipette connected to an electronic aspirator, after having centrifuged the sample for 10 minutes at 2000 rpm (Eppendorf, Centrifuge 5810). Moreover, during measurement, the separated sample liquid phase is stirred over a magnetic plate with a stir bar put directly into the water phase, which continuously spins it. Below pictures of mobile glove box and electrodes are reported.

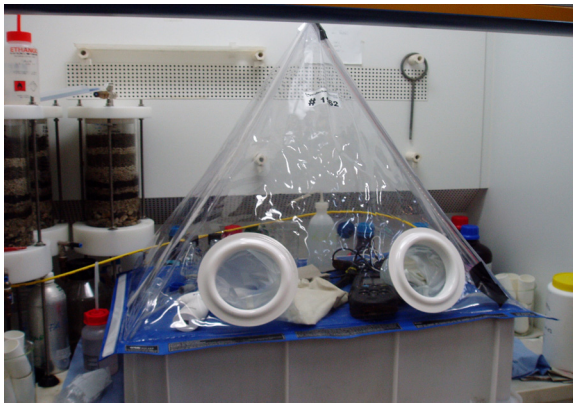


Figure 3.3 – Mobile glove box

Figure 3.4 – Electrodes kit

3.3.2 Dissolved organic carbon

The DOC measurement, concerning both cases, has been performed in two different ways during the experiments development.

The DOC measurement, concerning both (anaerobic and aerobic) experiments, has been performed in two different ways during the experiments development.

At the beginning, the DOC determination has been made with tests coming from *Hach Lange* Chemical Industries, listed with the identification code LCK 385 (validity range: 3 – 30 mg/l) and showed in the picture below.

Figure 3.5 – DOC Test

Once checked that pH and temperature values are acceptable for the test (that are 15 – 25 °C and pH range between 3 – 10), it is possible to perform the measure, following the instructions. In a first step all inorganic carbon is purged after acid addition. In a second step the remaining organic carbon is oxidised to CO_2 and consequently purged through a membrane into an indicator cell. Subsequently the colour change is measured on the photometer. (*Hach Lange*, spectrophotometer DR 2800).

However these tests are very expensive, so we decided to use instrumental measurement with a "LiquiTOC-System", (Elementar Analysensysteme, Germany). In this case, 20ml sample has to be prepared.

The validity range of the electronic reader device is 1 – 10 mg/l; if a dilution is needed, the rest of the test sample is filled up with Milli-Q-water (Millipore, MA, US), that is purified water with a specific resistivity of 18,2 MΩ .

The measurement is based on chemical oxidation, followed by purging of the produced CO_2 into a NDIR measurement cell. The instrument is externally calibrated with calciumhydrogenphtalate and samples are calculated based on this calibration.

3.3.3 Nitrate and sulphate

Nitrate and sulphate are measured with an Ion Chromatography ICS-900 from Dionex (Dionex, Sunnyvale, CA, US) equipped with an automated sampling device AS40 . Anions are separated on a packed column according to their retention on the filling material. The eluent is a mixture of sodium carbonate (8.0 mM) and sodium hydrogen carbonate (1.0 mM).The electronic IC device provides chromatograms, like the one presented in figure 3.6. Calibration is done with an external standard dilution series (1mg/l – 100mg/l) of Multi Component Anion Mix 2 purchased at VWR International (VWR, Darmstadt Germany) containing fluoride, cloride, bromide, nitrate, phospahte and sulphate. For integration, calibration and calculation Chromeleon software from Dionex is used.

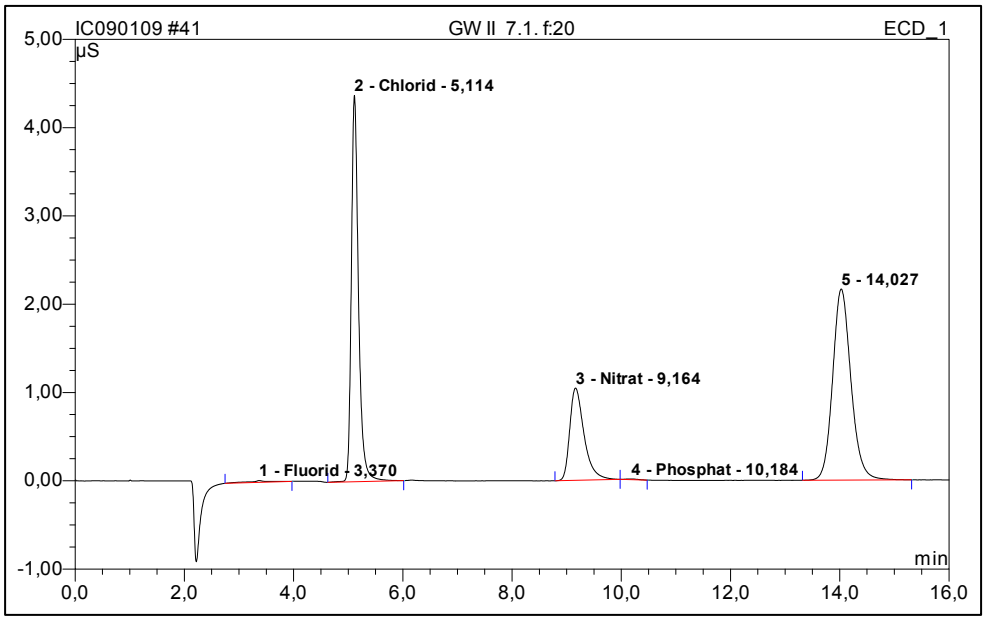


Figure 3.6 – IC chromatogram

3.3.4 Nitrite

The nitrite concentration is determined with a photometric method: nitrite forms a diazonium salt at pH 1.9 in the presence of 4-aminobenzoesulfonamide, that reacts with N-(1-naphthyl)-1,2-diaminoethane-dihydrochlorid and forms a pink colorant. The absorption of this colouring is measured at 540 nm. The measurement is done on a Tecan SPECTRAfluor Plus (Tecan Trading Ag, Switzerland). 3 identical tests are prepared (triplicates are needed to ensure a statistical validity), containing each 300µl of sample and 20µl coloring reagent solution,

The reagent solution contains:

- → 2 g of 4-Aminobenzosulfonamid
- → 25 ml of MQ water
- → 5 ml of orthophosphoric acid
- → 0,100 g of N-(-1-Naphthyl-1,2-Diaminoethan-Dihydrochloride)

Once that all samples are prepared, they have to be placed in an electronic shaker and mixed for 1 hour. Also some external standards must be prepared, used to set the calibration curve for every measurement.

3.3.5 Sulphite, sulphide, ammonia and phosphate

The determination of all these 4 ions is performed with photometric tests from *Hach Lange* Chemical Industries. In each test the measure procedure is reported, and we followed it literally. In the following table all tests are presented:

Substance (Test code)	Characteristic	
Sulphite (LCW 054)	The validity range for this test is 0,1 – 5,0 mg/l and acceptable ranges for T and pH are 15 – 25 °C and 3 – 10. In this case it is necessary to prepare a blank solution with distilled water, to identify the zero value for concentration.	
Sulphide (LCK 653)	The validity range in this case is 0,1 – 2 mg/l and acceptable ranges for T and pH are 15 – 25 °C and 3 – 10. No blank solution is needed.	
Ammonium (LCK 305)	This determination is performed only for the sulphate testing line, since other lines don't contain ammonium, and in the aerobic line. The validity range is 1,3 – 15 mg/l and acceptable ranges for T and pH are 18 – 22 °C and 4 – 9. Also in this case no blank solution is needed.	
Phosphate (LCK 349)	Phosphate determination is performed in both anaerobic testing lines (nitrate and sulphate) and in the aerobic line. The validity range is 0,05 – 1,50 mg/l and acceptable range for T and pH are 15 – 25 °C and 2 – 10.	

Table 3.14 – *Hach Lange* test for different chemical parameters

3.4 Toxicity Monitoring

In addition to chemical parameters monitoring, particular attention has been given to the assessment of ecotoxicological effects of contaminant soil on the bacteria. To investigate the toxicity of PAH contaminated soil, an aquatic bioassay based on a specific indicator organisms has been used. The testing microorganisms are *Vibrio fische*ri (identification code: NRRL-B-11177), a marine luminescent bacterium, which allows to make a rapid and sensitive evaluation of toxicity (Frische, 2002).

About 10% of the energy produced through the *Vibrio fische*ri metabolism is used to emit light at a specific wavelength, λ = 490 nm, and an inhibition of the metabolic process involves a lumiscence decrease (Scherr, 2004). This inhibition of luminescence may be used to investigate the effects of the contaminated sand on the bacteria metabolism, evaluating the toxic impact on the microorganisms.

The test procedure, for the determination of EC value, (LUMISTox, Luminescent bacteria test LCK 480, Dr. Bruno Lange GmbH & Co KG, Düsseldorf, Germany) requires a pH between 7 +/- 0,2 otherwise if pH is below 6,0 or above 8,0 pH-related light inhibition can occur. Serial dilution (1:1 (v/v), 1:4 (v/v), 1:8 (v/v), 1:16 (v/v), 1:32 (v/v)) of sample are prepared, including also a negative control with 2% NaCl. Triplicates of each dilution are prepared. Luminescence is measured before and after an incubation time of 30 minutes at 20°C with a Luminometer (LUMISTox LGP 259, Dr. Bruno Lange GmbH & Co KG, Düsseldorf, Germany). Previously, the initial bacterial luminescence is measured.

As reported in the in the instructions from Dr. Lange test, the inhibition percentage must be calculated as further showed:

$$I(\%) = \frac{I_{30C} - I_{30}}{I_{30C}} \cdot 100$$

where

$I(\%)$ = Luminescence inhibition in percentage;

I_{30} = Bacterial luminescence after 30 minutes;

I_{30C} = Theoretical bacterial luminescence after 30 minutes, without influence of toxic substances = $I_0 \cdot F_K$;

I_0 = Initial bacterial luminescence, before the incubation;

F_K = Temporary correction factor (average of the triplicates) = $\frac{I_{30,NaCl}}{I_{0,NaCl}}$;

$I_{30,NaCl}$ = Bacterial luminescence after 30 minutes in the sample added with 2% of NaCl;

$I_{0,NaCl}$ = Initial Bacterial luminescence, preceding the incubation, in the sample added with 2% of NaCl .

The more harmful effect on the microorganisms the higher is the percentage inhibition of the light emission,. At the end is possible to calculate the EC_{20} and EC_{50} values, that refer to the concentration of sample that causes 20% and 50% inhibition , respectively.

3.5 PAHs extraction

The PAH extraction is separated in two main parts, which correspond to the solid phase PAHs extraction, with the aim of determining the xenobiotic compounds still bounded to sand particles, and the water phase PAH extraction, which aims to investigate the dissolved and thus mobile PAH concentration. As mentioned before, for each sampling day, 3 replicates are analyzed for each testing line.

To separate the phases the samples must be filtrated over paper filters (Whatman 595½, Folded Filters, Ø 150mm). The whole operation is conducted under the fume hood. To ensure that every sand particle inside the sample flask was transported to the filter, the flask is repeatedly washed with distilled water. Below a picture of the filtration phase is reported.

Figure 3.7 – Filtration phase under the fume hood

3.5.1 Solid phase extraction

Concerning the solid phase extraction a *Soxhterm* apparatus (Gerhardt Soxhterm extractor model 2000 automatic, Bonn, Germany) is used. The solvent used during the extraction procedure is ethyl acetate. In contrast to classical Soxhlet extraction the thimble containing the sample is suspended in boiling solvent in a first extraction step. Subsequently the solvent is evaporated to a level just below the extraction thimble followed by a longer second extraction step with clean solvent dripping continuosly on the sample and thus ensure complete extraction.

The filtrated samples are input into the thimbles (40 x 85mm; Whatman International ltd., Maidstone, England) for Soxhterm extraction and then 85 ml of ethyl acetate, with the function of extraction solvent (Szolar et al., 2002). The Soxhterm apparatus has been programmed to start with a 60 minutes boiling phase of the sample being immersed in the solvent, followed by a 90 minutes Soxhlet extraction.

Figure 3.8 – Soxhterm Apparatus

A solvent reduction routine leads to a final volume equal to 20 – 25ml. The extract is then transferred to 50 ml volumetric flask and filled to the mark using the extraction solvent. Finally 1 ml vials for the HPLC analysis are prepared with two different dilutions in acetonitrile for each sample. The dilution ratios used are 1:50 (v/v) and 1:200 (v/v).

3.5.2 Liquid phase extraction

The liquid phase extraction begins when the filtration is finished, putting 10ml of dichloromethane (CH_2Cl_2) inside the mineral medium flask and shaking vigorously the flask for 1 minute. The use of dichloromethane is typical in liquid-liquid extraction, in which there is the passage of the solute from a solvent to another one, usually better soluble in the second one.

Extraction was forwarded with a separating funnel, showed in figure 3.9, shaking by hands, which allow splitting in 2 phases, aqueous one and organic one. Unpolar PAHs are more prone to solve in the less polar organic phase and thus can be separated from the water phase. The extraction step is repeated twice to ensure complete extraction of the contaminants.

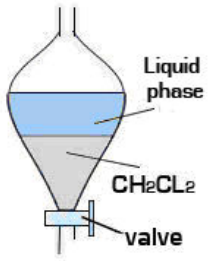


Figure 3.9 – Funnel for liquid phase extraction

Subsequently, the collected extract is evaporated in a Turbovap system (Zymark, Germany) and a solvent exchange to acetonitrile is accomplished. Through this evaporator workstation it is possible to run the process automatically for 6 simultaneous samples, fixing the end point at 0,8ml. The concentrator uses a patented gas vortex shearing action and optical sensors to provide fast and efficient evaporation of organic solvents.

Figure 3.10 – Turbovap evaporator

Every position is monitored by a photocell connected to an acoustic sensor, which indicates the expected achievement. Every time that the end point is reached for a single position, the nitrogen flow, necessary to evaporate the solvent, is stopped and a visual sensor indicates it. Hence the extraction is ended and prepared vials have to be analyzed by the HPLC device to obtain the real concentrations.

3.6 PAH measurement with HPLC

The high pressure liquid chromatography (HPLC) analysis technique is based on the principle that various mixture components are divided between two phases in a different way, concerning their affinity with both of them. The high pressure liquid chromatography allows to separate various compounds contained in a solvent, exploiting the affinity balance between a "stationary phase", placed inside the chromatographic column, and a "mobile phase", that flows through this column. A compound more affine with the stationary phase spends more time to go through the column, compared with a compound that is more affine with the mobile phase. Furthermore the method can use a gradient profile for the eluent ranging from polar to unpolar. The sample, is injected automatically into the chromatographic system, where is driven through the stationary phase by applying hundreds atmospheres pressures. After the column the samples passes through one or more detectors (e.g. IR, UV-VIS detector, mass spectrometer).

The device utilized is a Hewlett Packard 1050 series with a HP 1100 series 3d-FLD (Fluorescent Detector), an HP 1050 series DAD (Diode Array Detector) and an HP Autosampler. The instrument control and data processing is performed by agilent HP Chemstation.

The columns used are:

- → Pre-column: Vydac High Performance Guard Column, reverse phase C-18;
- → Working Column: C-18 Grace Vydac 201 TP 54 (250 x 4,6 mm, particle size 5μm).

The column held at 26°C and operates with an eluent flow-rate of 1,5mL; the eluent gradient profile is set up with 50% of Acetonitrile and 50% of Milli-Q-Water, as follow:

- → Starting with 50% Acetonitrile for 2,5 minutes;
- → Linear gradient of 9,5 minutes until 90% Acetonitrile;
- → Linear gradient of 8 minutes until 100% Acetonitrile, 2,5 minutes holding;
- → Subsequently a linear gradient (2,5min) back to initial conditions for column conditioning before the next run.

The time spent by the sample to elute, that pass through the column, is the *retention time* (RT). RT is a function of the investigated substance, but also of the chosen stationary and mobile phases. After separation the sample passes through the DAD to measure acenaphthylene at 310 nm and consequently through a 3- dimensional FLD, allowing the residual 15 PAHs to be measured at 4 excitation and emission pairs simultaneously. .The excitation wavelength is set to 260nm, but the emission wavelength varies between 350, 420, 440 and 500nm. The investigated substances release energy in a precise excitation/emission wavelength couple, as showed in the table 3.15.

PAH	Detector	Emission Wavelength (nm)
Acenaphthene	FLD	350
Phenanthrene	FLD	350
Fluoranthene	FLD	440
Pyrene	FLD	420
Benzo(a)pyrene	FLD	420

(Szolar, 2002)

Table 3.15 – Emission wavelength corresponding to different PAHs

The resulting chromatograms contain all 5 detector traces, showing the 16 EPA PAHs. The HPLC system is externally calibrated with a standard dilution series from 10 ppb to 800 ppb. For each HPLC sequence a new method is calibrated and the integration results of the chromatograms are then calculated taking sample weight and dry matter into account. Usually determination limit of the HPLC method is calculated based on calibration data, and ranges from 10 to 20ppb, and the maximum value is 800 ppb; if the results are not within this range a new dilution has to be prepared.

In figure 3.11 and figure 3.12 overlapping and separated chromatograms are displayed. In the first figure the 3rd traces are shown overlapping, whereas in the second one the single excitation/emission wavelength couple traces are reported. 3 chromatograms are displayed in this last figure due to only 3 wavelength couple are needed to detect the 5 PAHs used to contaminate the sand (see table 3.15).

On the x-axis the retention time is shown and on the y-axis the Absorbance Units (AU) are reported. The characteristic peaks of each detected substance are labelled with the compound's name.

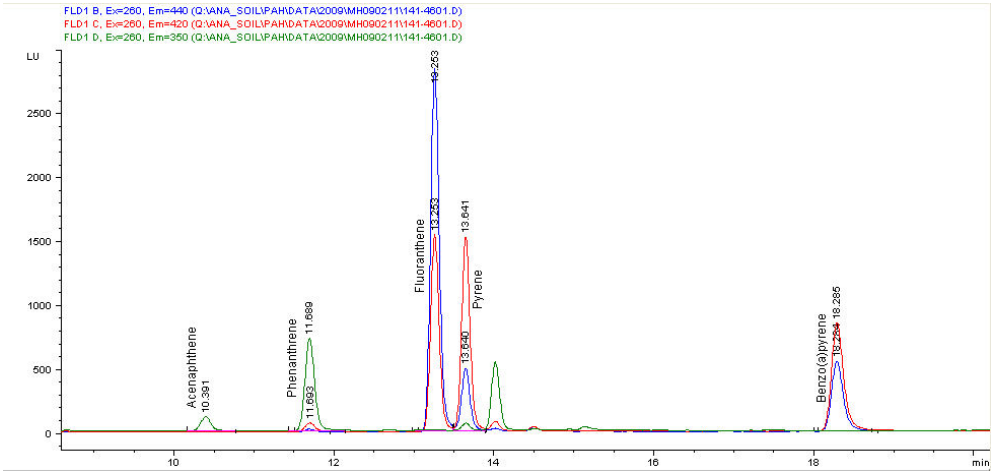


Figure 3.11 – HPLC overlapping chromatograms for PAH concentration determination

As mentioned, there are 3 replicates for each sampling days: the final results are given by the average (μ) of 3 measurements, and also the standard deviation (σ) is calculated. To be sure that the result is reliable the coefficient of variation (CV) is calculated, and it must be less than 10 to consider the result acceptable. To calculate this CV the following formula is used:

$$CV = \sigma \cdot 100 / \mu$$

For the out-layer analyses the Grubbs Test was applied.

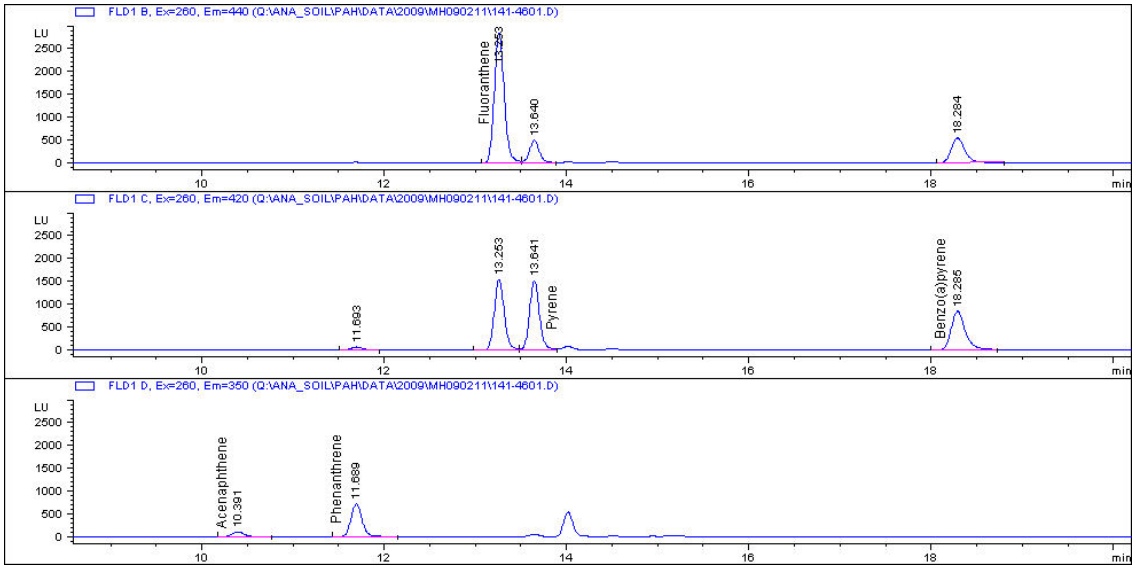


Figure 3.12 – HPLC separated chromatograms for PAH concentration determination

Chapter 4

Results

1.2 Sand characterization

4.1.1 Dry Matter

The dry matter measurement has been useful to calculate the exact amount of sand to put in each sample, in order to avoid the presence of water, as explained in paragraph 3.2.1.1.

Dry matter of sand spiked with PAH solution was measured to be 99,61% for anaerobic and 99,76% for aerobic experiment.

Basing on these data, it is possible to calculate the correct amount of sand to add in each sample to have exactly 10g of dry sand. The formula to calculate the real weight is the following:

$$W_R = {}^{W_T}\!/_{DM}$$

where:

W_R = real weight of sand in each sample;

W_T = theoretical weight of sand, equal in both experiment to 10g.

Therefore the weight obtained to put inside each sample is:

Experiment	Real sand weight
Anaerobic	10,039g
Aerobic	10,024g

Table 4.1 – Real sand weight for anaerobic and aerobic experiment

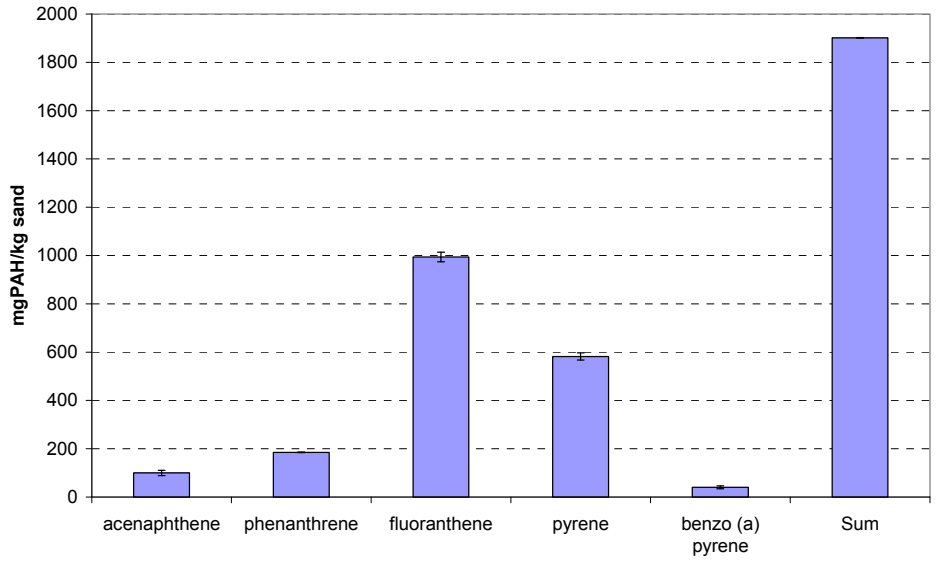
4.1.2 Spiking results

After the sand contamination, 3 replicates have been analyzed by the HPLC analysis, obtaining the results reported in table 4.2 and displayed in figure 4.1. The considered recovery percentages have been nearly respected, even though, generally, the values have been lower than the expected averages. The only exception has been the acenaphthene, for which the recovery obtained has been 62,11% instead of the predicted 23,67%. The other recovery percentages have been: 82,43% for phenanthrene, 85,88% for fluoranthene, 89,78% for pyrene and 71,61% for benzo(a)pyrene. Due to the lower recovery, the total contamination amount detected by the HPLC analysis has been about 1901,5 mg PAH / kg sand, instead of 2000 mg PAH / kg sand, as expected since the beginning.

PAH	Average concentration (mg PAH / kg sand)	(SD)
Acenaphthene	99.7	(10.50)
Phenanthrene	185.4	(1.09)
Fluoranthene	994.1	(19.37)
Pyrene	581.9	(14.93)
Benzo(a)Pyrene	40.4	(5.86)
SUM	**1901.5**	**(0.95)**

Standard deviation (SD) has been calculated on three replicates

Table 4.2 – PAHs concentration of spiked sand (Day 0)



→ The black bar represents the standard deviation (n=3)

Figure 4.1 – PAHs concentration of spiked sand (Day 0)

4.2 Anaerobic degradation results

4.2.1 PAH content in sand phase

The determination of PAH concentration is carried out by HPLC analysis, with different dilution steps, considering different concentration of polycyclic compounds. For each sampling day 3 replicates have been analyzed. The dilution rates chosen for further HPLC analysis of PAH content in sand are 1:50 (v/v) and 1:200 (v/v). In table 4.3 and in figure 4.2 the concentration of the 5 PAHs sum is displayed for the whole experiment length. In table 4.3, the PAHs sum concentration is reported in mg of PAH per kg of sand.

Qualitatively no notable reduction over the incubation time was noticed in samples analysis, with the PAH content remaining nearly constant to the starting value of about 2000 mg PAH/kg sand under both biotic (nitrate and sulphate conditions) testing lines. In case of abiotic control poisoned with $HgCl_2$, no chemical or physical phenomena has been detected to cause pollutant losses from the system and the concentration has been detected close to 2000 mg of PAH per kg of sand over the incubation time.

All data reported in this paragraph respect the statistical validity standard, since coefficient of variance is lower than 10.

Day	1	(SD)	34	(SD)	55	(SD)
Nitrate testing line	2030.57	(3.19)	1720.79	(12.34)	1963.17	(34.77)
Sulphate testing line	1935.55	(54.07)	1624.97	(2.22)	1789.83	(55.27)
Abiotic control	1862.68	(21.15)	ND	ND	1925.00	(28.65)

Day	83	(SD)	111	(SD)	130	(SD)
Nitrate testing line	1984.42	(153.67)	2100.85	(91.36)	2076.45	(49.34)
Sulphate testing line	2068.16	(10.72)	2021.14	(135.20)	2241.06	(85.16)
Abiotic control	ND	(ND)	2092.83	(147.98)	2104.4	(12.45)

ND = not detected value

Table 4.3 – Concentration in mg PAH/kg sand of 5 PAHs sum in solid phase; anaerobic experiment

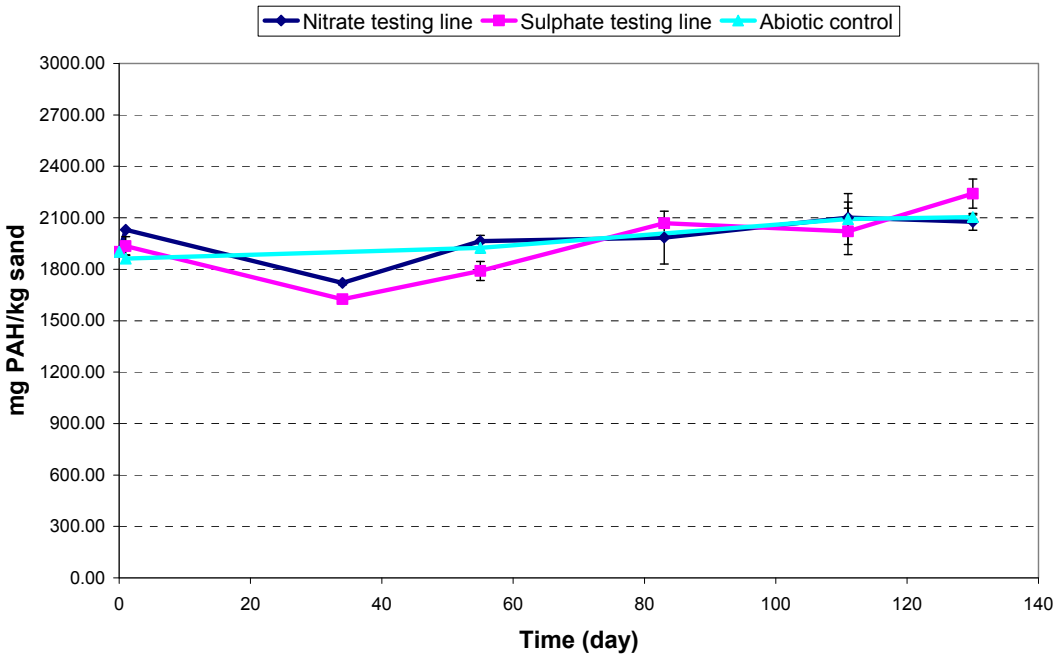


Figure 4.2 – Concentration trend of 5 PAHs sum in solid phase; anaerobic experiment

The sum trend is representative also of the average concentration trend for each PAH. Qualitatively analysis has elucidated no significant variations in the sand contamination, suggesting no biodegradation of contaminants under anaerobic conditions.

In the following figures, the concentration trend in the sand phase for each PAH is reported, showing the data from the three testing lines, the two biotic and the one abiotic. Values are presented in several tables at the end of the section.

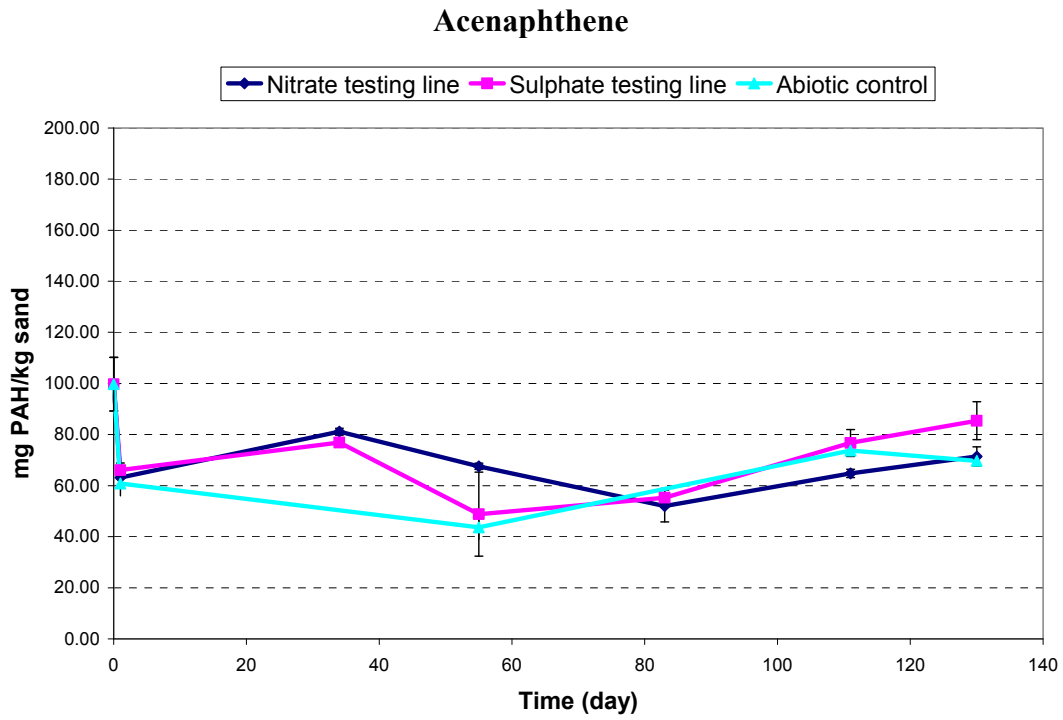


Figure 4.3 – Concentration trend of acenaphthene in solid phase; anaerobic experiment

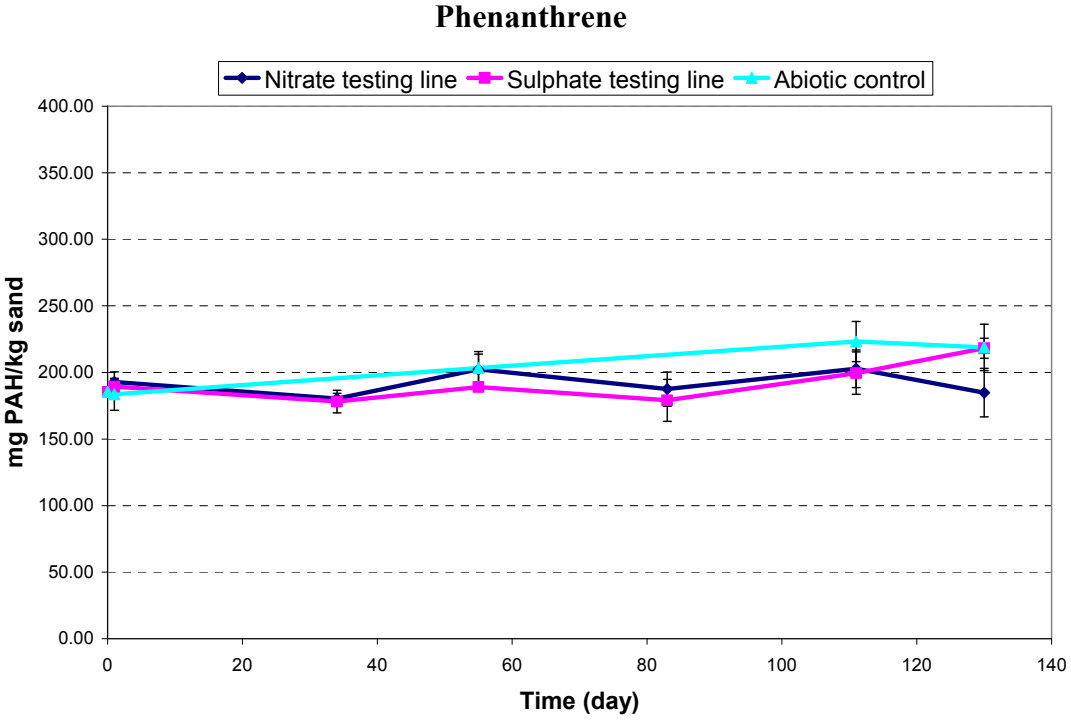


Figure 4.4 – Concentration trend of phenanthrene in solid phase; anaerobic experiment

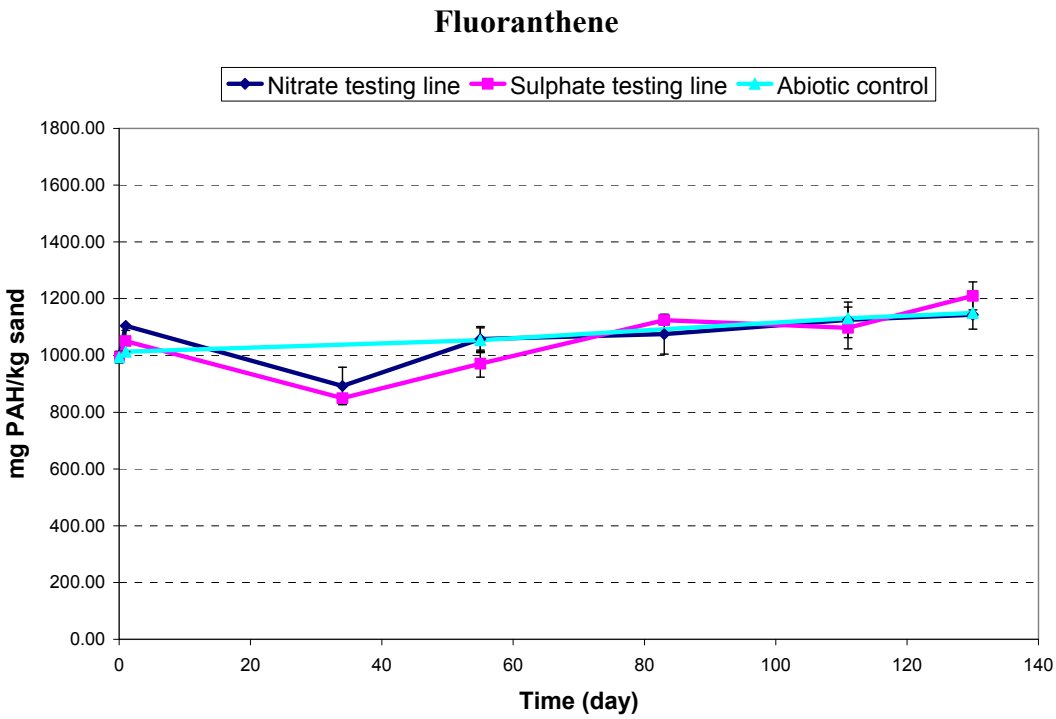


Figure 4.5 – Concentration trend of fluoranthene in solid phase; anaerobic experiment

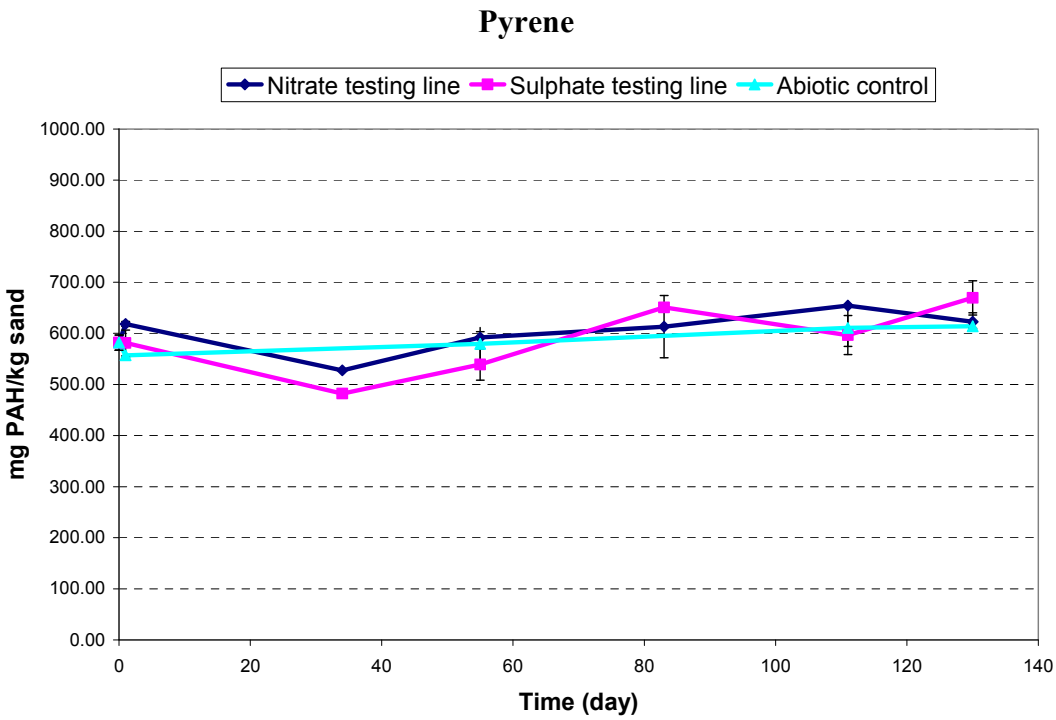


Figure 4.6 – Concentration trend of pyrene in solid phase; anaerobic experiment

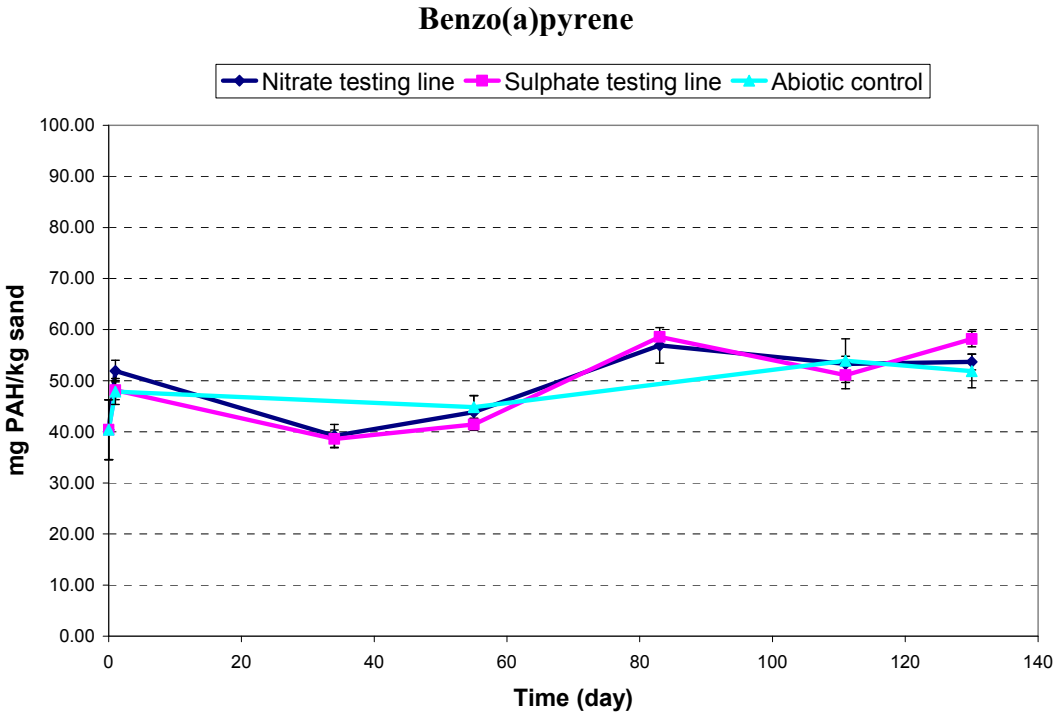


Figure 4.7 – Concentration trend of benzo(a)pyrene in solid phase; anaerobic experiment

Nitrate reducing conditions testing line

Day	0	(SD)	1	(SD)	34	(SD)	55	(SD)	83	(SD)	111	(SD)	130	(SD)
acenaphthene	99.74	(10.50)	63.20	(0.49)	81.11	(1.34)	67.54	(1.22)	52.00	(6.23)	64.81	(1.64)	71.40	(3.79)
phenanthrene	185.41	(1.09)	192.81	(7.66)	180.29	(3.94)	202.35	(13.34)	187.48	(12.89)	202.80	(14.16)	184.86	(18.17)
fluoranthene	994.07	(19.37)	1104.13	(3.01)	892.38	(66.10)	1057.61	(43.92)	1074.91	(70.08)	1125.63	(62.46)	1143.63	(50.52)
pyrene	581.91	(14.93)	618.52	(5.11)	527.80	(1.40)	591.83	(11.56)	613.11	(60.97)	654.29	(1.70)	622.89	(17.43)
benzo(a)pyrene	40.41	(5.86)	51.91	(2.11)	39.21	(2.25)	43.84	(3.23)	56.92	(3.49)	53.32	(1.46)	53.68	(1.53)

Sulphate reducing conditions testing line

Day	0	(SD)	1	(SD)	34	(SD)	55	(SD)	83	(SD)	111	(SD)	130	(SD)
acenaphthene	99.74	(10.50)	66.05	(2.81)	76.87	(1.46)	48.85	(16.46)	55.26	(3.67)	76.69	(5.29)	85.41	(7.41)
phenanthrene	185.41	(1.09)	189.33	(5.73)	178.10	(8.46)	189.09	(3.16)	179.04	(15.70)	199.53	(15.96)	218.16	(7.50)
fluoranthene	994.07	(19.37)	1050.75	(37.41)	849.28	(10.04)	971.19	(47.80)	1124.46	(16.74)	1097.14	(73.86)	1209.76	(48.97)
pyrene	581.91	(14.93)	581.26	(25.15)	482.13	(0.92)	538.93	(30.55)	650.82	(4.12)	596.67	(38.24)	669.56	(33.58)
benzo(a)pyrene	40.41	(5.86)	48.17	(1.86)	38.60	(1.73)	41.46	(1.19)	58.58	(0.54)	51.11	(2.67)	58.17	(1.53)

Abiotic control testing line

Day	0	(SD)	1	(SD)	55	(SD)	111	(SD)	130	(SD)
acenaphthene	99.74	(10.50)	60.84	(4.82)	43.66	(4.69)	73.75	(4.55)	69.689	(4.86)
phenanthrene	185.41	(1.09)	183.60	(12.02)	203.38	(10.41)	223.26	(15.13)	218.86	(17.49)
fluoranthene	994.07	(19.37)	1013.21	(0.49)	1053.69	(43.66)	1131.11	(88.28)	1149.9	(5.33)
pyrene	581.91	(14.93)	557.16	(4.04)	579.45	(31.95)	610.80	(36.44)	614.02	(3.85)
benzo(a)pyrene	40.41	(5.86)	47.88	(2.54)	44.83	(2.30)	53.91	(4.28)	51.898	(3.30)

Concentration is reported in mg PAH/kg sand – Standard deviation in brackets)

Table 4.4 – Acenaphthene, phenanthrene, fluoranthene, pyrene and benzo(a)pyrene concentrations for the anaerobic experiment

4.2.2 PAH concentration in the liquid phase

PAH concentrations of the liquid phase have been obtained by HPLC analysis, utilizing a different dilution compared with the sand phase. Particularly, a dilution step 1:100 (v/v) has been used. Figures 4.8 and 4.9 displays the detected PAH concentrations for the whole experiment duration.

At the beginning of the experiment the PAH concentration detected for the three testing lines was about 20 mg/l, equal for each line (see table 4.5). As displayed in figure 4.9 the abiotic control is the only testing line which shows a stable concentration, varying between 23 and 42 mg/l, remaining lower than the other two testing lines for the whole experiment duration.

Day	1	(SD)	34	(SD)	55	(SD)
Nitrate testing line (mg/l)	21.3	(5.29)	30.65	(3.81)	52.17	(6.06)
Sulphate testing line (mg/l)	13.89	(2.84)	47.77	(14.67)	41.68	(17.39)
Abiotic control (mg/l)	23.12	(2.50)	ND	(ND)	41.23	(5.97)

Day	83	(SD)	111	(SD)	130	(SD)
Nitrate testing line (mg/l)	92.23	(2.64)	101.70	(30.98)	43.38	(15.64)
Sulphate testing line (mg/l)	84.67	(14.22)	47.39	(13.52)	21.23	(2.36)
Abiotic control (mg/l)	ND	(ND)	32.62	(7.63)	37.03	(15.55)

ND = not detected value

Table 4.5 – Concentration in mg/l of 5 PAHs sum in liquid phase; anaerobic experiment

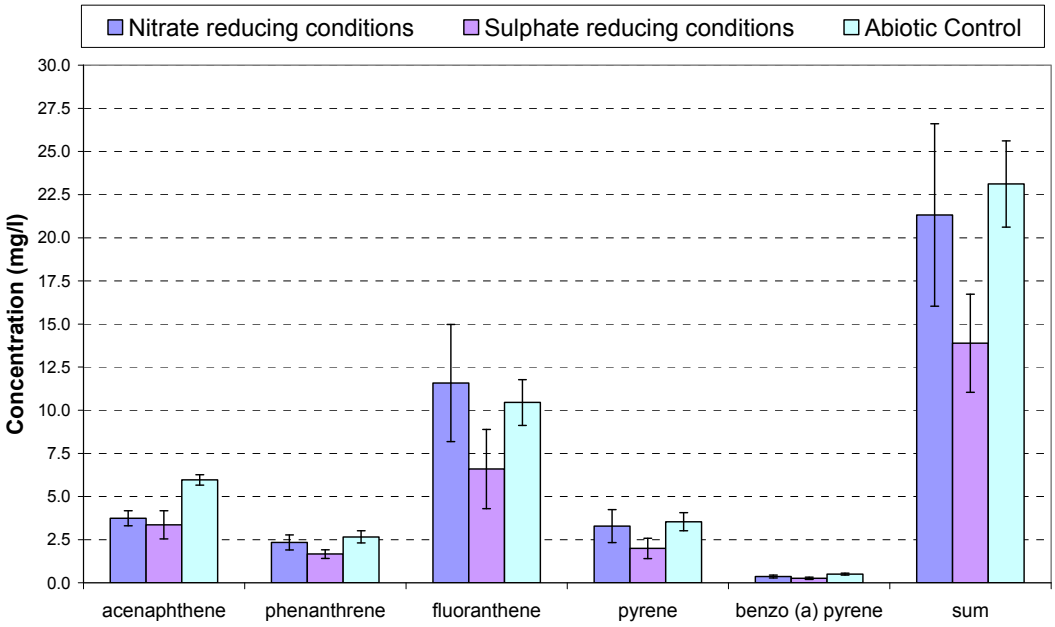


Figure 4.8 – Concentration of 5 PAHs sum in liquid phase at day 1; anaerobic experiment

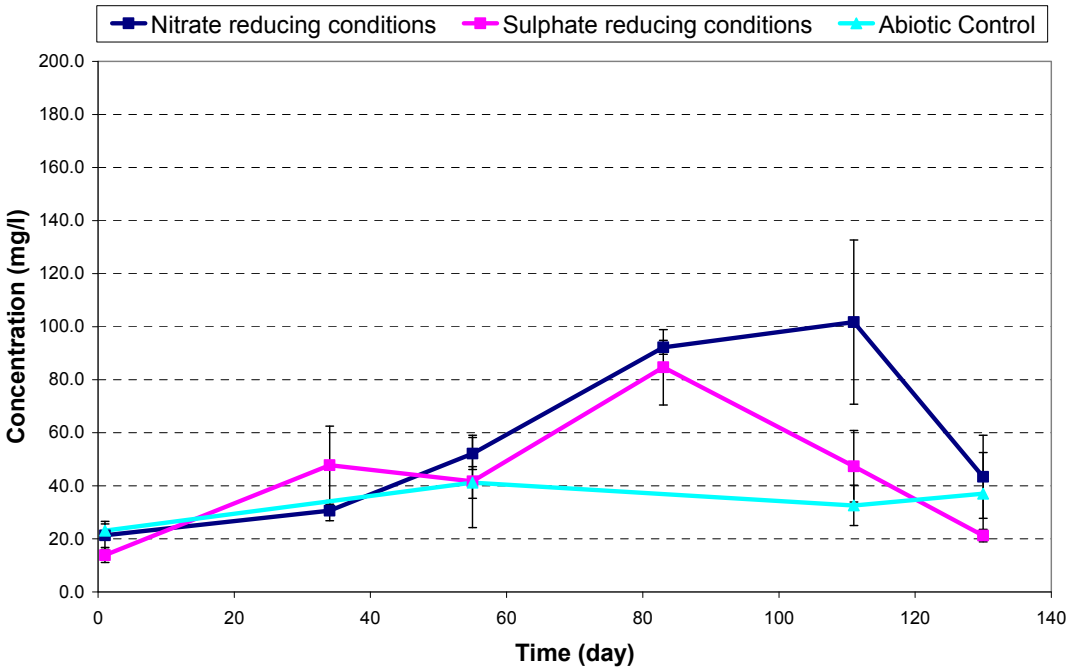


Figure 4.9 – Concentration trend of 5 PAHs sum in liquid phase; anaerobic experiment

Concerning the nitrate and sulphate reducing conditions testing lines, significant variations are showed and in day 83 values equal to 5 times the starting PAH concentrations has been reached.

Due to the relevant results fluctuation, an additional work has been carried out in sampling days 83 and 111: liquid phase extraction has been split in two parts and mineral medium has been extracted and analyzed separately from the distilled water, used to wash the sample flasks before the filtration. Results are displayed in figure 4.10. In table 4.6, mineral medium phase and water phase refer to PAH concentration in mg/l in the respective phase; washing water indicates the amount of distilled water used to wash the flask in grams (the values are average of three samples). As displayed in the last column (excess), a quantity varying, between the 17% and the 41%, of the total PAH concentration detected in the liquid phase analysis derives from the washing with distilled water. The amount of distilled water used is not directly linked to the PAH increase in liquid phase, suggesting that the physical process connected to the washing out is random. For the abiotic control this phenomenon seems to be less relevant than for the biotic testing lines.

Day	Mineral medium phase (mg/l)	Water phase (mg/l)	Washing water (g)	Excess (%)
Nitrate reducing conditions				
83	53.73 (0.92)	38.50 (1.72)	37.44 (4.10)	41.7
111	52.25 (2.17)	14.07 (3.43)	26.29 (1.79)	21.2
Sulphate reducing conditions				
83	68.36 (10.70)	16.31 (3.52)	29.55 (4.77)	19.3
111	34.45 (11.65)	12.94 (2.14)	32.37 (5.81)	27.3
Abiotic control				
111	26.87 (4.08)	5.75 (0.58)	30.79 (3.18)	17.6

Standard deviation in brackets

Table 4.6 – Concentration in mg/l of 5 PAHs sum in mineral medium and water phase; anaerobic experiment, day 83 and day 111

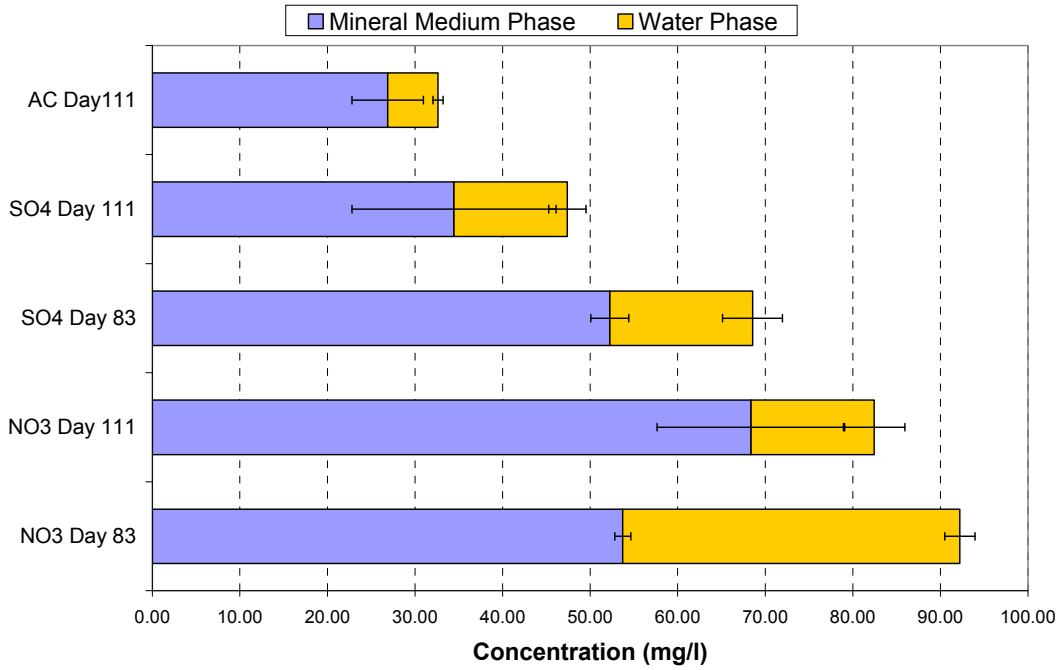


Figure 4.10 – Concentration in mg/l of 5 PAHs sum in mineral medium and water phase; anaerobic experiment

4.2.3 Chemical parameters trend

4.2.3.1 Oxidation reduction potential trend

The oxidation reduction potential, as described in chapter 1, is a very important parameter, ruling the anaerobic biodegradation, since particular degradation situations are favoured with different ORP values. As reported in table 4.7 and showed in figure 4.11, for abiotic control the values are around 400 mV, even though it has been not possible to obtain stable values with the electrodes.

Concerning the two biotic testing line redox potential differs a lot, with variation of more than 100% over the incubation time.

Nitrate testing line – In the first 32 days, the nitrate testing line redox potential swings between 100 and 200 mV. After the first microorganisms input (day 33, 100µl of anaerobic PAHs degrading bacteria from the stock culture), in the next sampling day no big changes have been detected on the ORP. However, negative values have been reached in day 83, after the input in day 75 of 1 ml of sludge coming from the water waste treatment plant, suggesting the development of good

conditions for degradation (for sludge characteristics see paragraph 3.2.3.1). But on day 111 the ORP value was back again to more than 150 mV, achieving the 200 mV in the last sampling day.

Sulphate testing line – In the first 5 weeks, redox potential in the sulphate reducing conditions testing lines has been similar to the nitrate conditions testing line, even though values swing between 40 and 150 mV. After the bacteria input, in day 55, negative values for ORP, typical for sulphidogenic biodegradation, have been achieved. Nevertheless, in the following sampling days, the ORP started to increase again, reaching positive values in the last 2 sampling days. The last sampling day value is not representative because oxygen has been detected inside the flask (see paragraph 4.2.3.3).

Day	Nitrate testing line (mV)	SD	Sulphate testing line (mV)	SD	Abiotic Control [a] (mV)	SD [b]
1	114.35	5.59	42.20	7.64	400	N.S.
7	162.85	5.73	96.43	20.57	430	N.S.
14	190.5	13.58	120.25	9.55	412	N.S.
21	162.5	2.26	146.25	3.89	ND	ND
24	110	0.00	101.95	11.67	ND	ND
27	98.5	10.61	91.55	12.66	ND	ND
30	155.3	0.00	100.80	13.01	ND	ND
55	209.65	9.83	-210.95	7.85	443	N.S.
83	-11.53	6.83	-15.67	3.79	ND	ND
111	162.50	12.64	56.77	8.60	400	N.S.
130	200.03	5.40	180.10	0.00	ND	ND

[a] ND when the value is not been detected
[b] N.S. when the value was not stable

Table 4.7 – Oxidation reduction potential for the anaerobic experiment

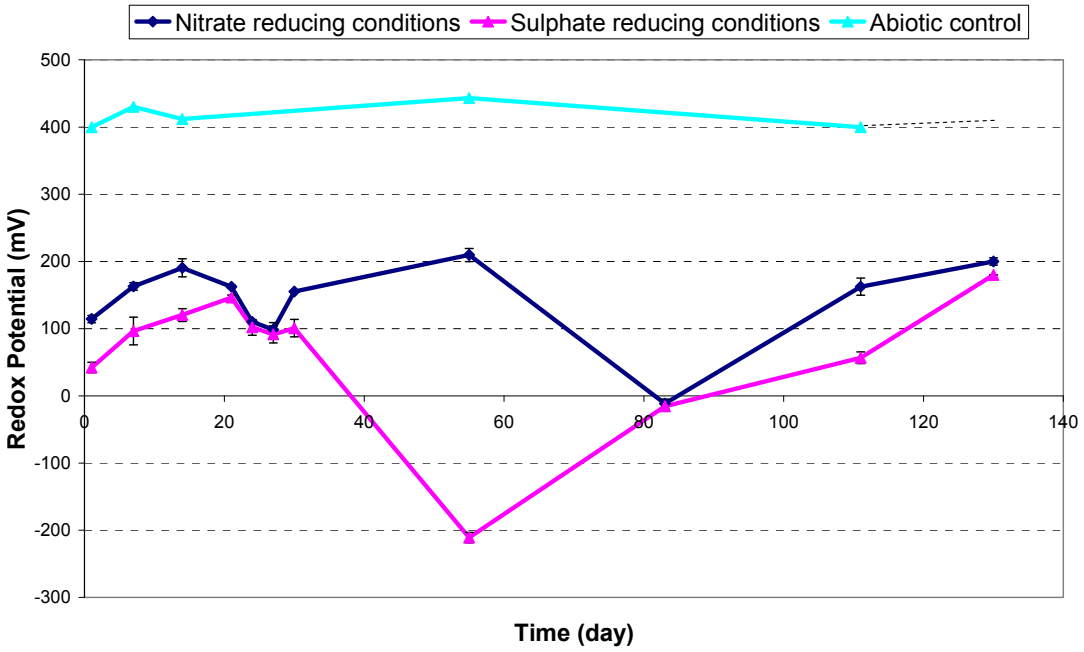


Figure 4.11 – Oxidation reduction potential for the anaerobic experiment

4.2.3.2 pH trend

In the following section, pH values for the anaerobic experiment testing lines are displayed (table 4.8 and figure 4.12). For nitrate and sulphate reducing conditions, the initial pH detected was low to allow bacteria activity and only a small decrease in the first 3 weeks has been detected.

On day 25, 4ml of a buffer solution (pH 7), have been added to the three testing lines to correct pH values. On day 27, pH values were closed to neutrality. These conditions have been considered as optimal for bacteria activity, due to the data from literature (paragraph 1.3.4.2).

After the buffer solution input, no significant pH variations have been detected in two biotic testing lines.

In case of abiotic control, the buffer solution has been also added to be sure to have the same status of biotic conditions.

Day	Nitrate testing line	SD	Sulphate testing line	SD	Abiotic Control	SD
1	6.00	0.08	6.68	0.01	5.77	0.01
7	5.71	0.03	6.65	0.05	5.90	0.03
14	5.47	0.02	5.98	0.01	6.14	0.01
21	5.91	0.02	6.01	0.01	ND	ND
27	6.81	0.00	7.02	0.01	ND	ND
30	6.80	0.01	7.09	0.00	ND	ND
34	7.05	0.01	7.09	0.01	ND	ND
55	6.99	0.01	7.17	0.02	6.73	0.04
83	7.23	0.01	6.87	0.01	ND	ND
111	7.49	0.01	7.04	0.02	6.71	0.01
130	7.35	0.01	7.18	0.02	6.47	0.02

Table 4.8 – pH for the anaerobic experiment

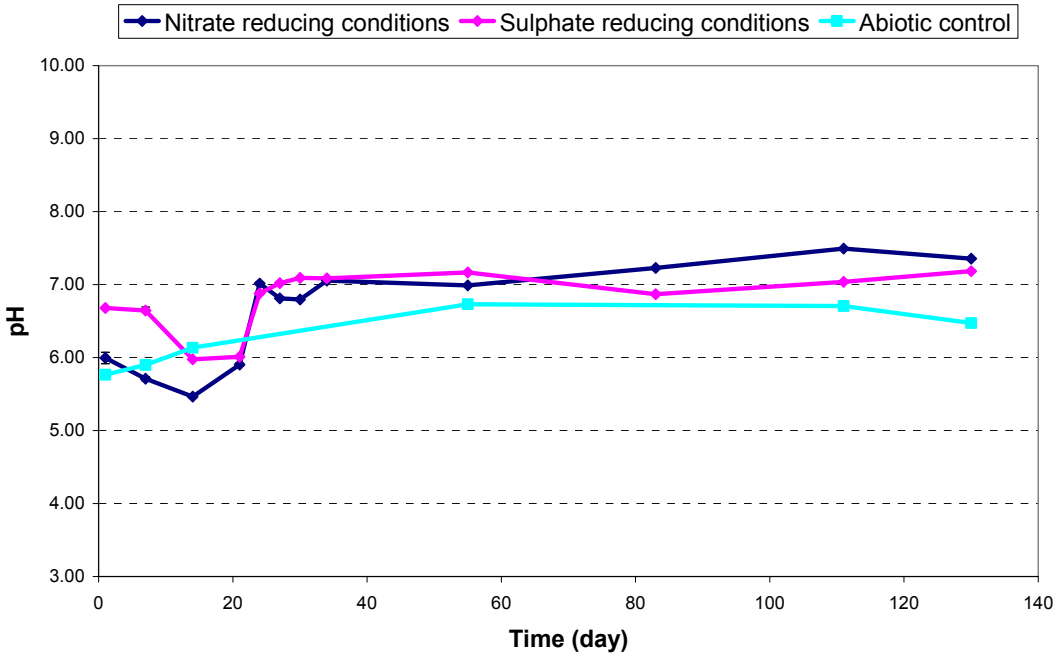


Figure 4.12 – pH variations in the anaerobic experiment

4.2.3.3 Oxygen concentration trend

The oxygen concentration has been monitored over the whole incubation time with an oxygen detection electrode. Since the beginning the concentration has been close to zero, as displayed in figure 4.13, suggesting the formation of anoxic conditions.

On day 14, high oxygen concentration has been detected for the abiotic control, probably caused by a not correct closing of the sample. Similar situation happened in the last sampling day for the sulphate reducers testing line. For the nitrate reducing conditions testing line, the oxygen level has been always satisfying to guarantee anoxic biodegradation.

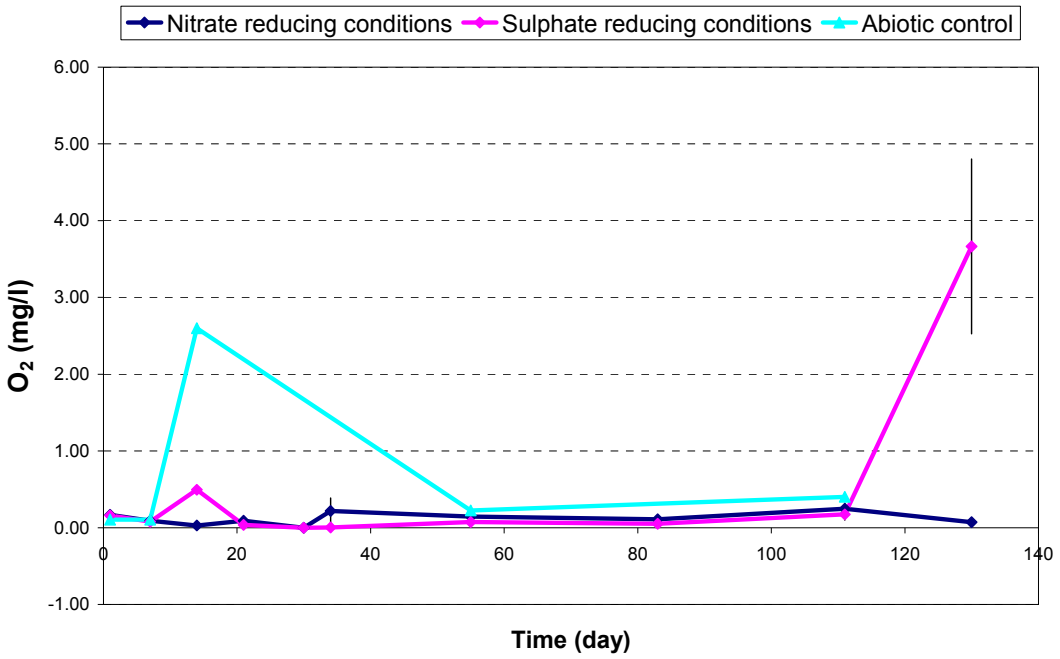


Figure 4.13 – Oxygen concentration in the anaerobic experiment

4.3.2.4 Temperature trend

Temperature is another important parameter involved in the biodegradation process (see paragraph 1.3.4.1). For this experiment, the room temperature has been assumed to be the working temperature for microorganisms, according to the aim of individualizing optimal conditions for real working case. In fact in field applications, it is easier to influence the other chemical parameters than the temperature.

The temperature obtained before and after the inoculation are reported in figure 4.14. The working values were detected between 20 and 23 °C.

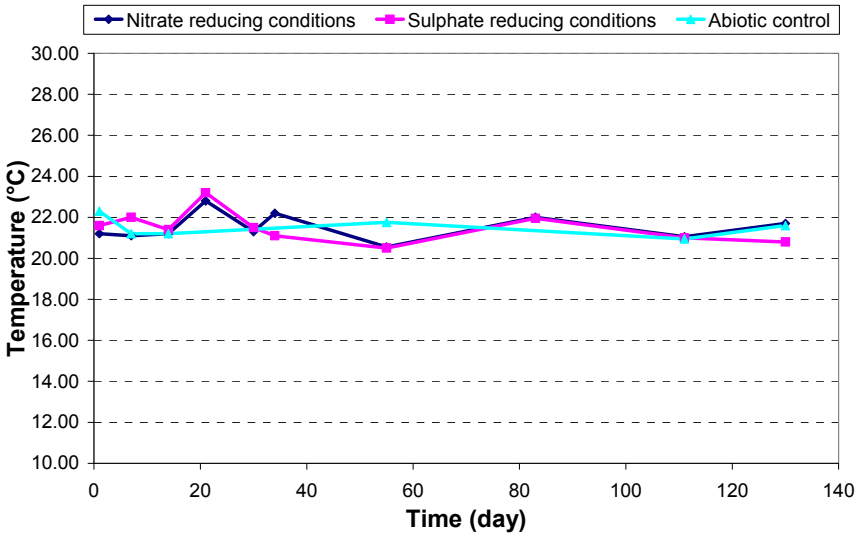


Figure 4.14 – Temperature trend; anaerobic experiment

4.2.3.5 Phosphate concentration trend

Another important parameter to monitor is phosphate, due to its importance for the microorganisms growth and a consumption of this compound is parallel to a microbial activity.

The starting concentration in the system, in accordance with mineral medium chemicals concentration (paragraph 3.2.2), is about 40 mg/l. After the input of buffer solution to correct the pH, phosphate concentration increased in both testing line to values around 400 – 450 mg/l for nitrate reducing conditions and 450 – 500 mg/l for sulphate reducing conditions. For the remaining duration of experiment, relevant variations in phosphate concentration have not been detected. The concentration is displayed in figure 4.15 and table 4.9. The points at day 0 indicate the theoretical concentration of mineral medium.

Day	Nitrate testing line (mg/l)	(SD)	Sulphate testing line (mg/l)	(SD)
34	430.19	1.34	480.83	3.73
55	432.61	2.30	486.69	3.73
83	397.13	33.63	468.48	2.78
111	401.70	2.85	469.62	3.06
130	425.86	2.16	450.28	1.66

Table 4.9 – Phosphate concentration in both biotic anaerobic testing lines

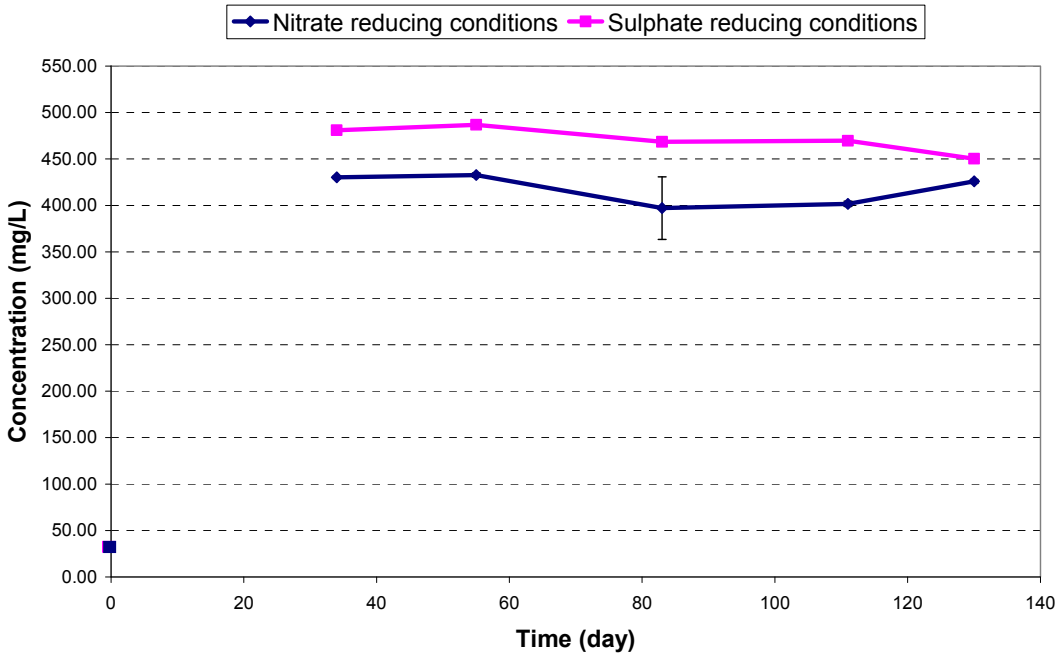


Figure 4.15 – Phosphate concentration in both biotic anaerobic testing lines

4.2.3.6 Nitrate concentration trend

In case of nitrate testing line was important to monitor the variation of nitrate concentration to predict the biodegradation and to relate the biodegradation rate with nitrate decrease. Since the nitrate was the only alternative electron acceptor present inside the microcosm, the biodegradation must be parallel to a nitrate use by bacteria and to the biotransformation of nitrate to gaseous nitrogen.

As displayed in table 4.10 and in figure 4.16, a final decrease equal to 21,7% of the initial concentration has been detected, suggesting a microbial activity inside the system.

Day	1	34	55	83	111	130
NO_3 (mg/l)	3414.30	3062.83	2916.26	2687.32	2543.40	2670.70
(SD)	0.00	5.12	10.36	219.87	23.85	19.86

Table 4.10 – Nitrate concentration in nitrate reducing conditions testing line

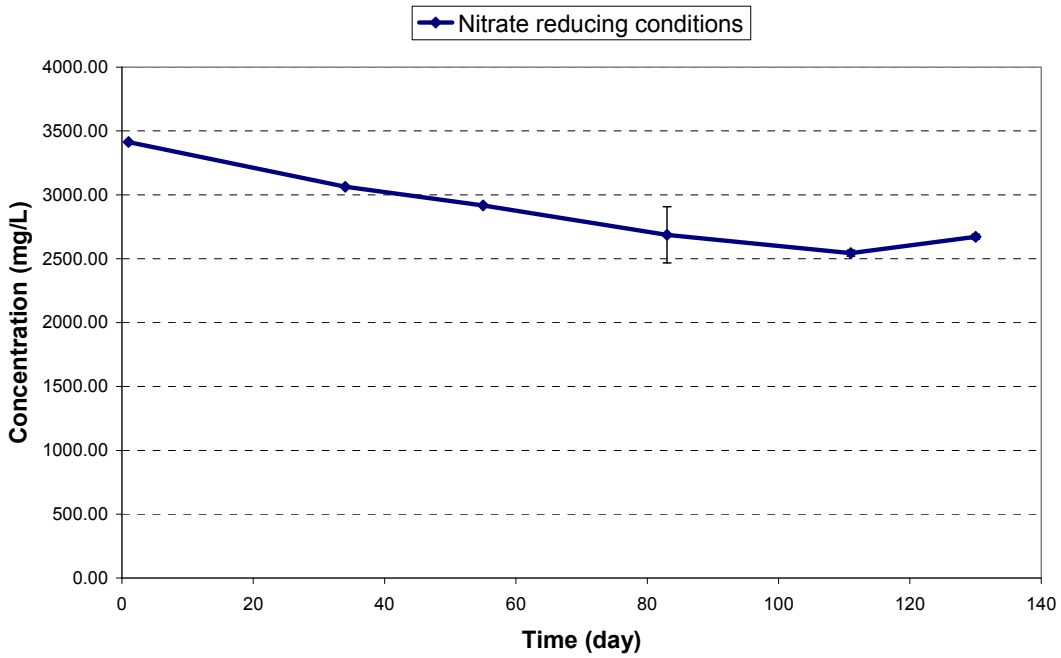


Figure 4.16 – Nitrate concentration in nitrate reducing conditions testing line

4.2.3.7 Nitrite concentration trend

As mentioned, the nitrite concentration has been monitored over the experiment time. The results obtained are showed in table 4.11 and figure 4.17. Measurement of nitrite was started at the day 55, since anaerobic bacteria have been added at the day 33 and no formation of nitrite was expected at the sampling day 34. From day 55 till the last sampling day, a production of nitrite is showed, suggesting a mild bacterial activity, according also with the nitrate depletion (paragraph 4.2.3.5).

Rittman and Mc Carty (2001) mention the oxidation-reduction which leads to nitrite formation starting from nitrate, even though the energy yields by the reaction is lower than the normal oxidation-reduction from nitrate to gaseous nitrogen. For this reason, the increase of concentration of this intermediate compound in the microcosm may be interpreted as a suffered nitrate reduction activity by bacteria, connected to a low energy production and which did not allow the development of proper conditions for PAH biodegradation.

The broken line indicates that the beginning NO_2 concentration was thought to be equal to zero.

Day	55	83	111	130
NO_2 (mg/l)	87.86	107.59	293.06	156.27
(SD)	0.33	0.24	3.20	1.60

Table 4.11 – Nitrite concentration in nitrate reducing conditions testing line

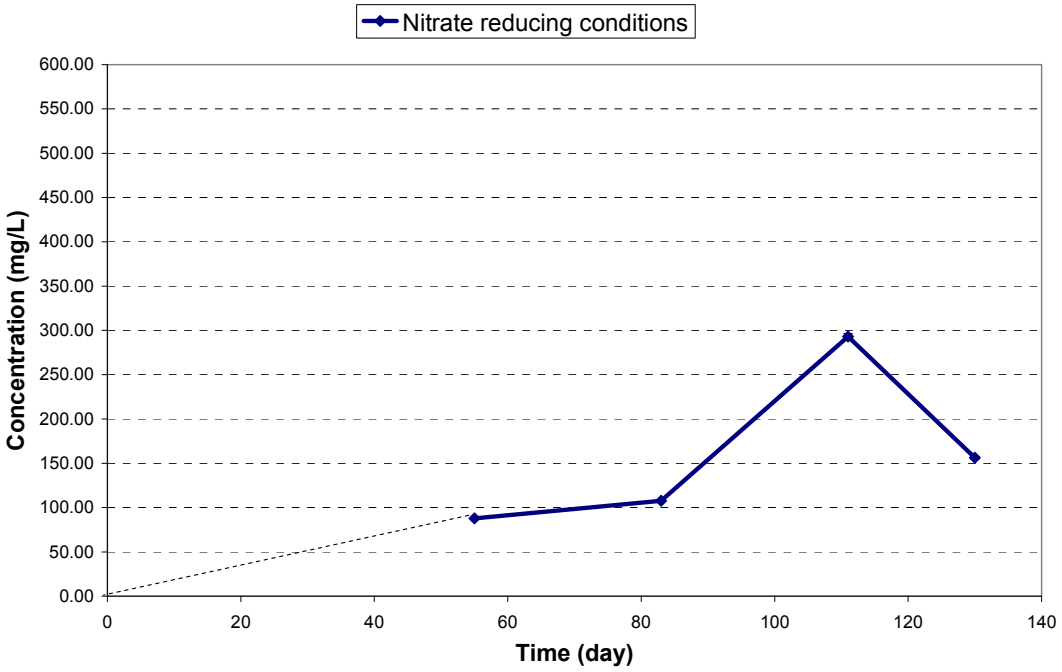


Figure 4.17 – Nitrite concentration in nitrate reducing conditions testing line

4.2.3.8 Sulphate concentration trend

In the sulphate reducing conditions testing line, monitoring the sulphate concentration allows to predict the biodegradation, since the sulphate is the only alternative electron acceptor present. During the development of the experiment, a 12% initial decrease, from the starting value of 3417 mg/l, has been observed in the first 4 weeks; however after this period no variations on sulphate concentration have been detected, suggesting that no degradation takes place in the system.

The results are showed in table 4.12 and in figure 4.18. Each value is the average of three replicates measurement.

Day	1	34	55	83	111	130
SO_4 (mg/l)	3146.92	2779.11	2800.96	2795.54	2810.17	2790.08
(SD)	0.00	20.87	325.60	17.71	15.42	22.45

Table 4.12 – Sulphate concentration in sulphate reducing conditions testing line

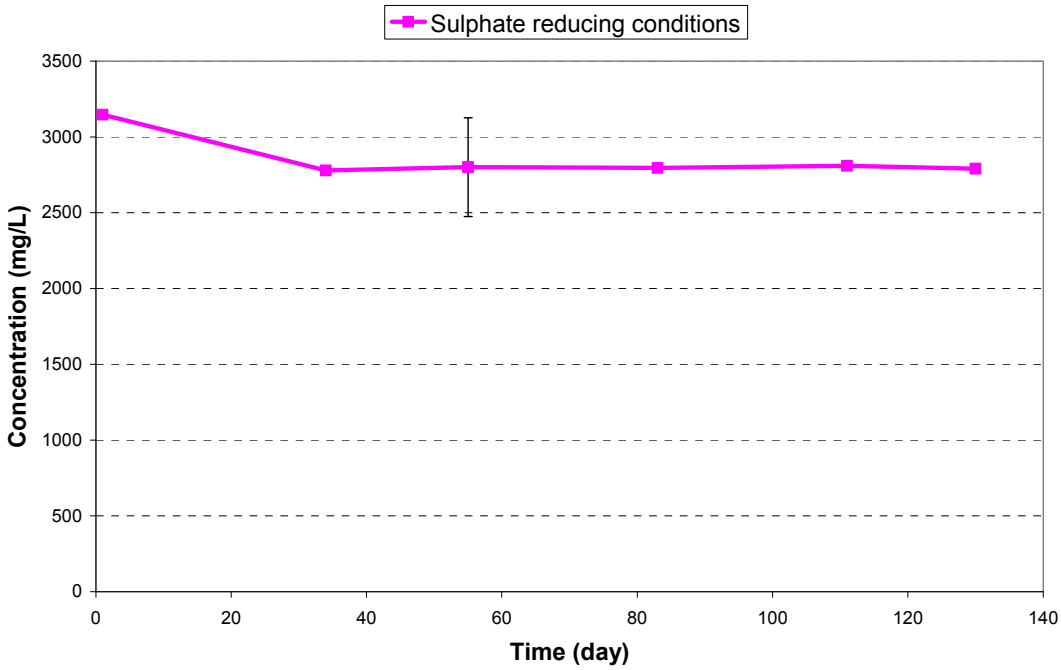


Figure 4.18 – Sulphate concentration in sulphate reducing conditions testing line

4.2.3.9 Sulphite and sulphide concentration trends

Over the duration of experiment, both sulphite and sulphide concentration have been monitored, but very low concentrations have been detected, according with the absence in sulphate consumption by bacteria.

After 55 days, no significant levels of sulphite have been detected through the analysis (~1 mg/l). Till the end of the experiment, no relevant variations are displayed in the samples (figure 4.19), suggesting sulphite was not present in the samples. The broken line indicates that the starting SO_3^{2-} concentration is though to be zero, since no sulphite has been present in the mineral medium.

In case of sulphide from day 55 till the end of the experiment no sulphide has been detected in the microcosm.

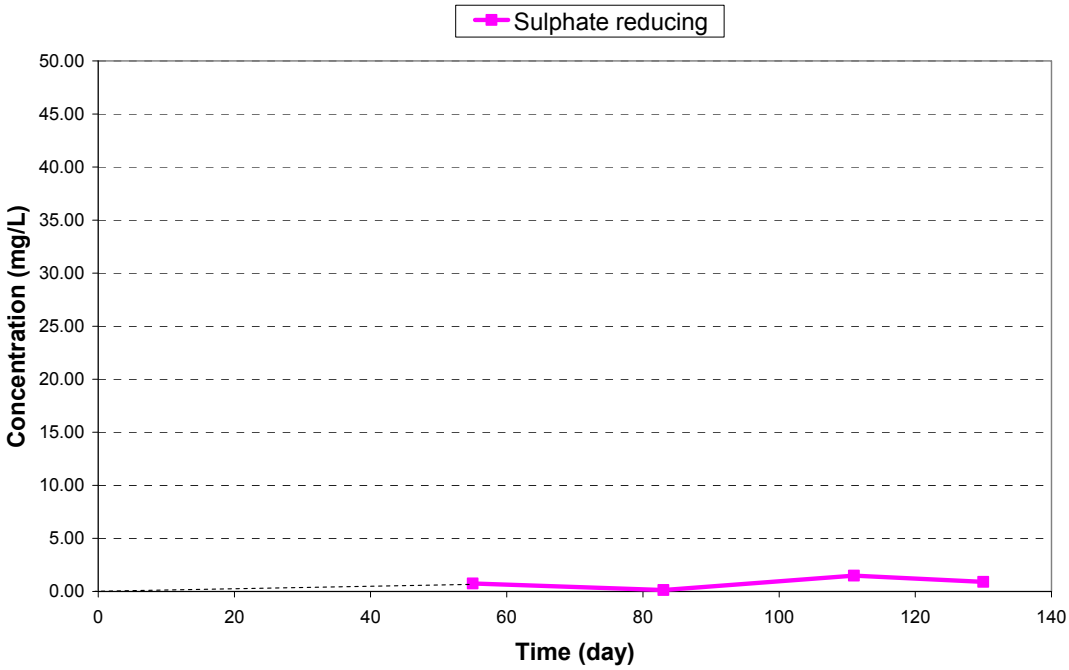


Figure 4.19 – Sulphite concentration in sulphate reducing conditions testing line

4.2.4 Toxicity monitoring

Lumistox test was used to determine acute toxicity of samples, after a 30 minutes incubation time. Both nitrate and sulphate reducing conditions testing lines have been sampled in the first day, in the day after the anaerobic microorganisms input (day 34) and in the last sampling day (day 130). Besides inhibition at different dilution rates, for each sample the EC_{50} value has been calculated, indicating the PAH concentration in liquid phase which causes a 50% inhibition of luminescent bacteria activity.

A high bacteria inhibition (> 99,9%) have been detected in day 130 for both nitrate and sulphate reducing conditions testing lines, even though it was suspected to be caused by a high turbidity of samples, which influences the results. For this reason it has been not possible to calculate the EC_{50} values for the last sampling day.

No inhibition has been calculated for abiotic testing line, because it was though to be equal to 100% since the beginning, due to the presence of mercury chloride.

The results are displayed in table 4.13 and reported in figure 4.20.

	Inhibition (%)		EC_{50} (mg/l)	
Day	1	34	1	34
Nitrate reducing conditions	73.8	72.9	4.45	5.79
Sulphate reducing conditions	68.0	72.3	3.61	9.80

Inhibition % refers to pure sample

Table 4.13 – Toxicity values for nitrate and sulphate testing line; anaerobic experiment

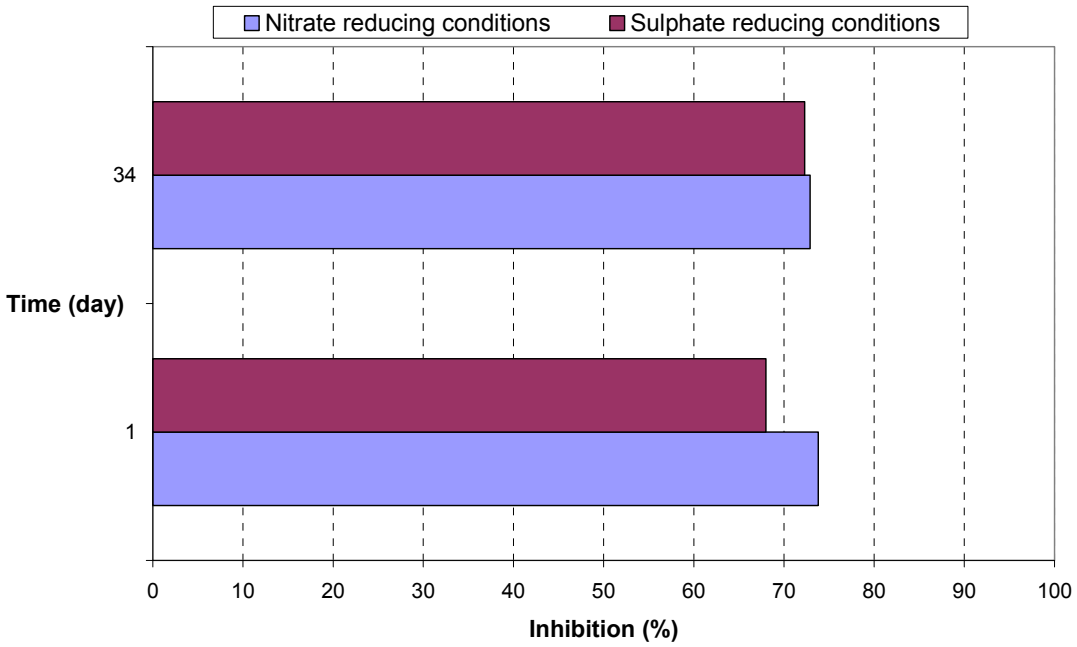


Figure 4.20 – Inhibition (%) for nitrate and sulphate testing lines; anaerobic experiment

In table 4.13, inhibition percentage refers to dilution 1:1 and indicates the amount of luminescent bacteria inhibited after 30 minutes incubation time. Whereas the EC_{50} concentrations indicate the liquid phase sample amount needed to cause an inhibition of 50% of luminescent bacteria.

According to Scherr (2004), a value for inhibition lower than 20%, under the chosen dilution step, does not imply an ecotoxicologic potential.

In day 1, nitrate testing line shows a higher inhibition (73.8%) than the sulphate testing line (68.0%), even though the difference is very low. After the

microorganisms input, the nitrate test showed a very small decreasing toxicity (-1%), whereas the sulphate test showed a small increasing toxicity (+ 4%). However, the difference reported may be due to the observational error. By the way, after day 34 both testing line display the same inhibition towards luminescent bacteria.

4.3 Aerobic degradation results

4.3.1 PAH content in sand phase

The aerobic experiment, as described, is carried out similarly to the anaerobic one, obtaining the PAH content by HPLC analysis with different dilution step. 3 replicates have been analyzed to give statistical effectiveness. The dilution rates chosen for further HPLC analysis have been 1:50 (v/v) and 1:200 (v/v). In table 4.14 and in figure 4.21 the results for the 5 PAHs sum are reported. In table 4.14 the PAH concentration is reported in mg PAH/kg sand.

On a quality level, no significant degradation has been detected over the incubation time in the aerobic conditions testing line, revealing no microbial activity, since no relevant differences are shown between the biotic line and the abiotic control. In case of acenaphthene, as displayed in figure 4.22, after 21 days an almost 80% decrease of the starting value has been detected. Subsequently the acenaphthene content turns to non detectable values in the last sampling days. However this decrease must be entirely attributed to abiotic effects and particularly to volatilization, since it has been detected in the biotic and abiotic testing line and both in sand and liquid phase, revealing no adsorption phenomena.

For all the other PAHs, relevant abiotic phenomena have not been detected over the incubation time, with the PAH concentration constant over the whole experiment length. Only in case of benzo(a)pyrene the decrease in the abiotic control must be attributed to the problems occurred to the samples (see paragraph 4.3.1.1).

Day	1	(SD)	7	(SD)	14	(SD)
Aerobic testing line	1803.6	(173.91)	1841.55	(64.75)	1881.70	(45.54)
Abiotic control	1996.44	(180.31)	ND	(ND)	ND	(ND)

Day	21	(SD)	55	(SD)	83	(SD)
Aerobic testing line	1761.71	(12.98)	1663.50	(76.86)	1848.49	(98.63)
Abiotic control	1944.57	(36.78)	ND	(ND)		

ND = not detected value

Table 4.14 – Concentration in mg PAH/kg sand of 5 PAHs sum in solid phase; aerobic experiment

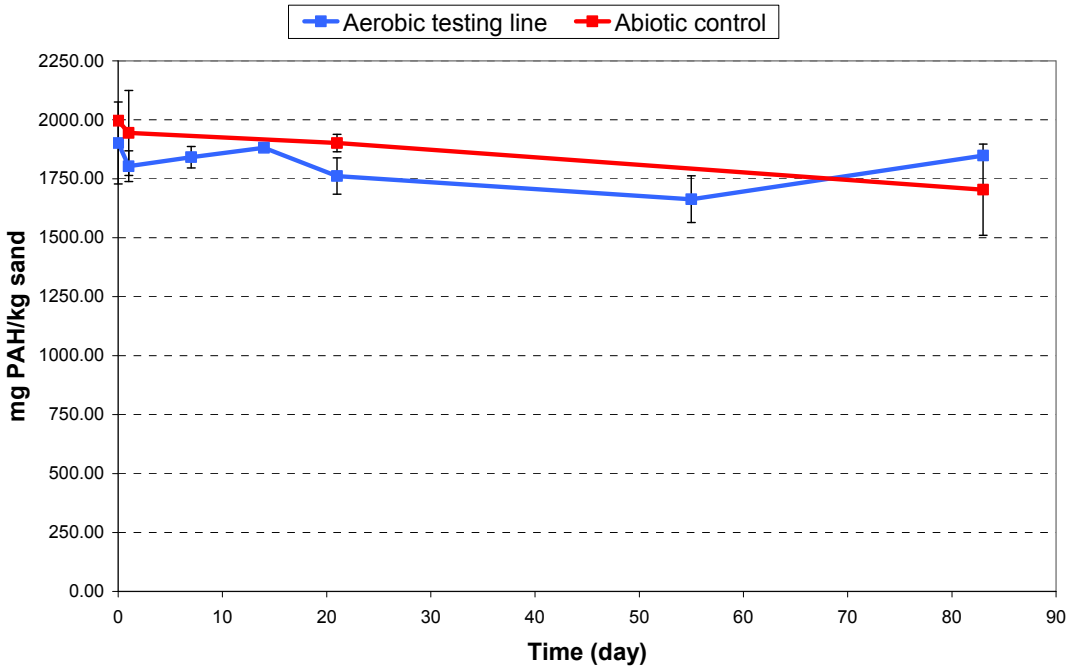


Figure 4.21 – Concentration trend of 5 PAHs sum in solid phase; aerobic experiment

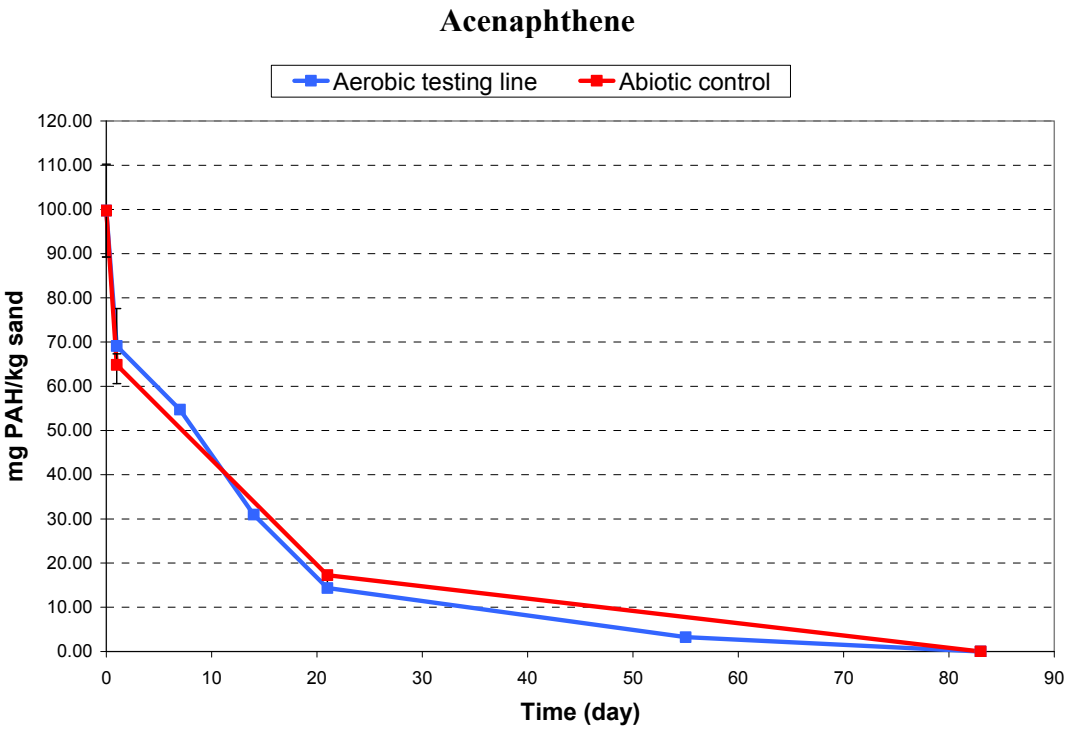


Figure 4.22 – Concentration trend of acenaphthene in solid phase; aerobic experiment

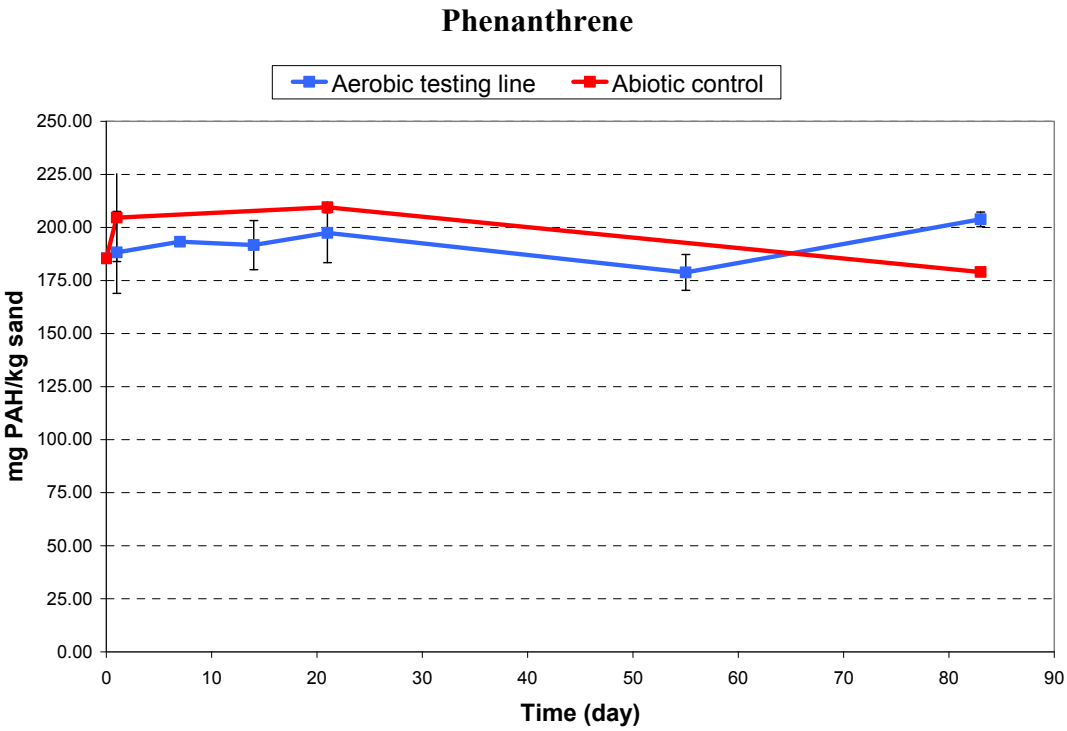


Figure 4.23 – Concentration trend of phenanthrene in solid phase; aerobic experiment

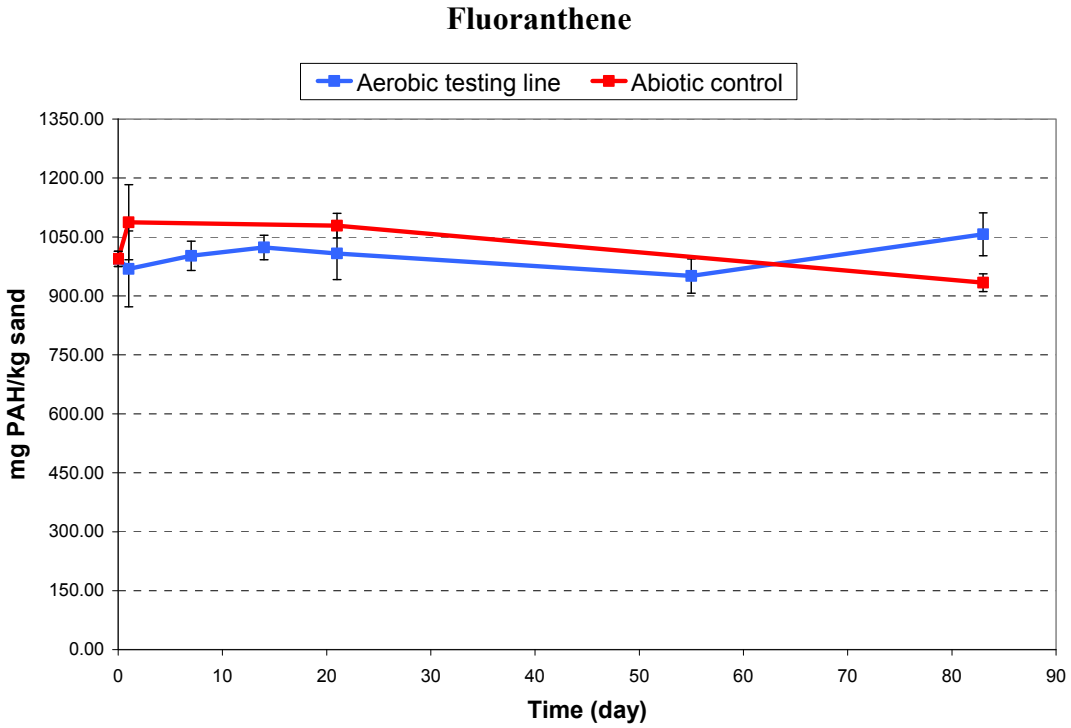


Figure 4.24 – Concentration trend of fluoranthene in solid phase; aerobic experiment

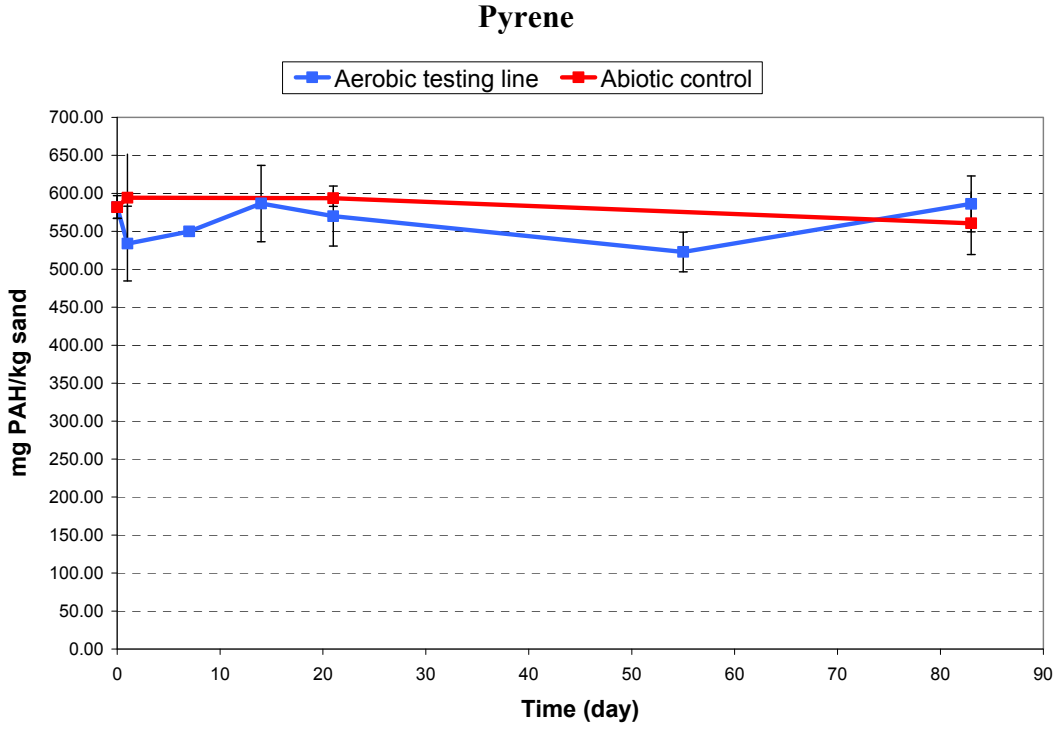


Figure 4.25 – Concentration trend of pyrene in solid phase; aerobic experiment

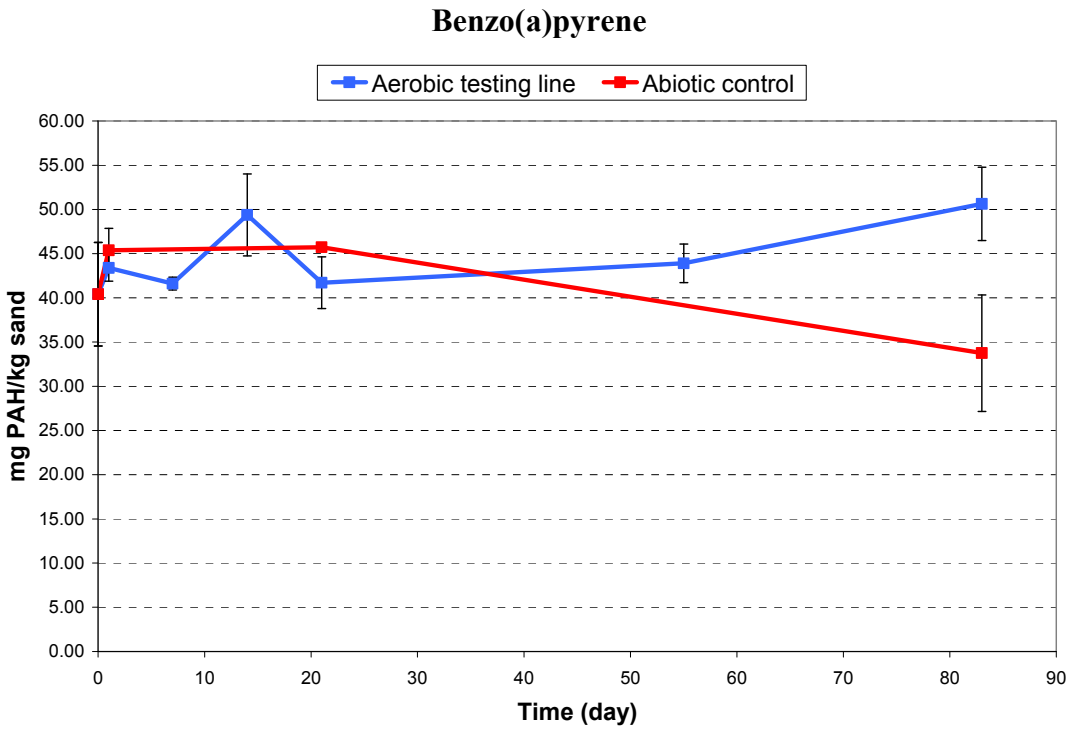


Figure 4.26 – Concentration trend of benzo(a)pyrene in solid phase; aerobic experiment

Aerobic conditions testing line

Day	1	(SD)	7	(SD)	14	(SD)	21	(SD)	55	(SD)	83	(SD)
acenaphthene	69.1	(8.49)	54.73	(0.07)	30.97	(0.88)	14.36	(0.20)	3.24	(0.53)	NDV	(NDV)
phenanthrene	188.2	(19.29)	193.29	(0.47)	191.69	(11.61)	197.43	(13.98)	178.85	(8.46)	203.84	(3.46)
fluoranthene	968.9	(96.27)	1002.00	(37.46)	1023.17	(31.04)	1008.29	(66.43)	950.77	(43.58)	1056.90	(54.71)
pyrene	533.9	(49.21)	549.92	(3.70)	586.49	(50.24)	569.96	(39.52)	522.76	(26.11)	586.07	(36.70)
benzo(a)pyrene	43.4	(1.48)	41.61	(0.72)	49.39	(4.63)	41.72	(2.92)	43.91	(2.17)	50.62	(4.14)

Abiotic control testing line

Day	1	(SD)	21	(SD)	83	(SD)
acenaphthene	64.83	(0.60)	17.27	(2.52)	0	(0)
phenanthrene	204.61	(20.69)	209.49	(2.58)	179.03	(0.59)
fluoranthene	1087.43	(95.35)	1078.60	(31.34)	933.87	(22.67)
pyrene	594.19	(57.34)	593.50	(10.69)	560.64	(41.24)
benzo(a)pyrene	45.38	(2.49)	45.72	(0.13)	33.744	(6.60)

Concentration is reported in mg PAH/kg sand – Standard deviation in brackets

NDV = not detectable value

Table 4.15 – Acenaphthene, phenanthrene, fluoranthene, pyrene and benzo(a)pyrene concentrations for the aerobic experiment

4.3.1.1 Problems occurred during the experiment

During the incubation time for aerobic experiment two problems occurred that may affect the analysis results.

First of fall, subsequently to the input of the spherical glass to increase the mixing in each sample and also better oxygen availability, a reduction in sand crystals size has been noticed, changing in this way the sand physical characteristic. Due to this breakage, a further suspension of lower weight particles appeared in the samples, giving a white colour to the mineral medium solution. Moreover, a deposit of fine particles size has been detected on the flask walls and close to the neck, which excludes a percentage of sand phase from biological processes. This phenomenon has been particularly relevant for abiotic testing line.

Secondly an undetermined loss of mineral medium has been detected in the last sampling day (day 83), caused by a malfunctioning of the shaker. A circa 50% loss has been estimated, which can seriously influences the results from water phase for the last sampling day.

The sample conditions are reported in figure 4.27 and 4.28.

Figure 4.27 – Sand deposit on flask neck and walls

Figure 4.28 – Undetermined loss of mineral medium in the last sampling day

4.3.2 PAH concentration in the liquid phase

With the liquid phase analysis, PAH content has been determined by HPLC analysis, with a dilution rate of 1:100. In figure 4.29 and in table 4.16 the results concerning the 5 PAHs sum concentration are displayed.

Day	1	(SD)	7	(SD)	14	(SD)
Aerobic testing line	60.0	(13.00)	59.97	(4.06)	78.91	(4.49)
Abiotic control	31.86	(1.73)	ND	(ND)	ND	(ND)

Day	21	(SD)	55	(SD)	83	(SD)
Aerobic testing line	68.06	(8.69)	158.37	(32.13)	53.05	(9.13)
Abiotic control	24.43	(7.08)	ND	(ND)	12.67	(0.87)

ND = not detected value

Table 4.16 – Concentration in mg PAH/kg sand of 5 PAHs sum in liquid phase; aerobic experiment

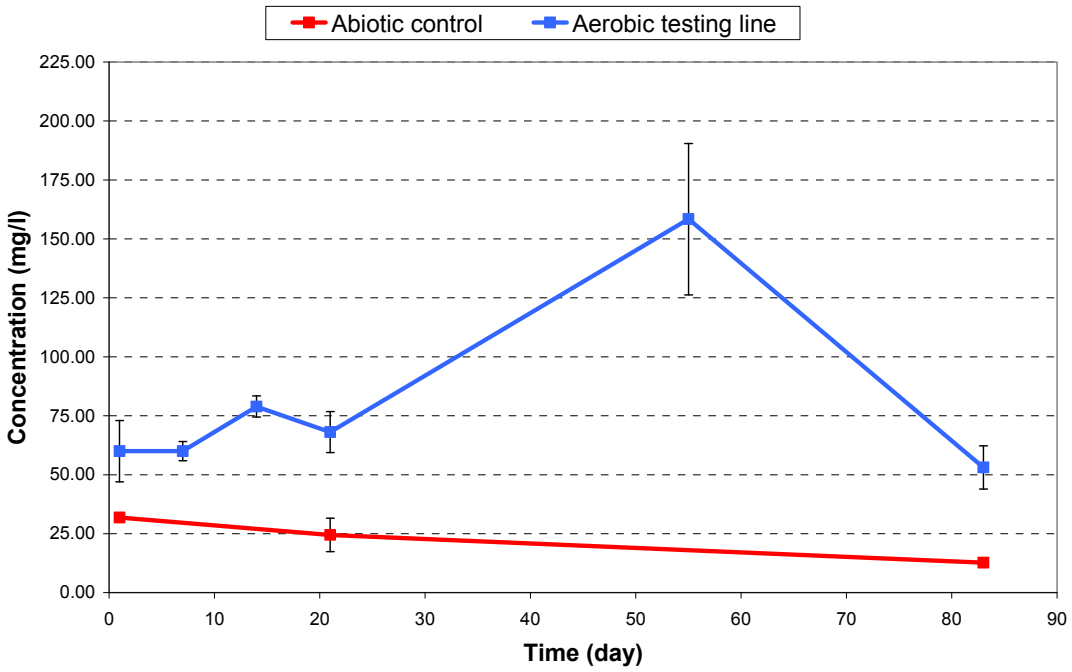


Figure 4.29 – Concentration trend of 5 PAHs sum in liquid phase; aerobic experiment

Since the beginning the detected organic compounds concentration has been higher than the expected one, from solubility data. The poisoned control displays 50% lower concentration than the aerobic testing lines, suggesting that the presence of mercury chloride inside the microcosm decrease the PAH solubility in water phase. Between

day 21 and day 55 an increase of 100% is shown, caused probably by the particles breakage (input of spherical glass). The significant decrease in the last sampling day, for both biotic and abiotic line, can not be considered significant, due to the problems occurred during the experiment progress.

In case of acenaphthene, according to the sand phase results, a decrease of 85% in the first 21 days has been detected in both testing lines, suggesting that abiotic phenomena occurred. From day 55 to the end of the experiment the acenaphthene concentration turned to non detectable values, indicating a physical removal efficiency of 100%. The principal phenomenon charged of acenaphthene removal should be volatilization. In figure 4.30 the acenaphthene concentration trend over the incubation time in the liquid phase is shown.

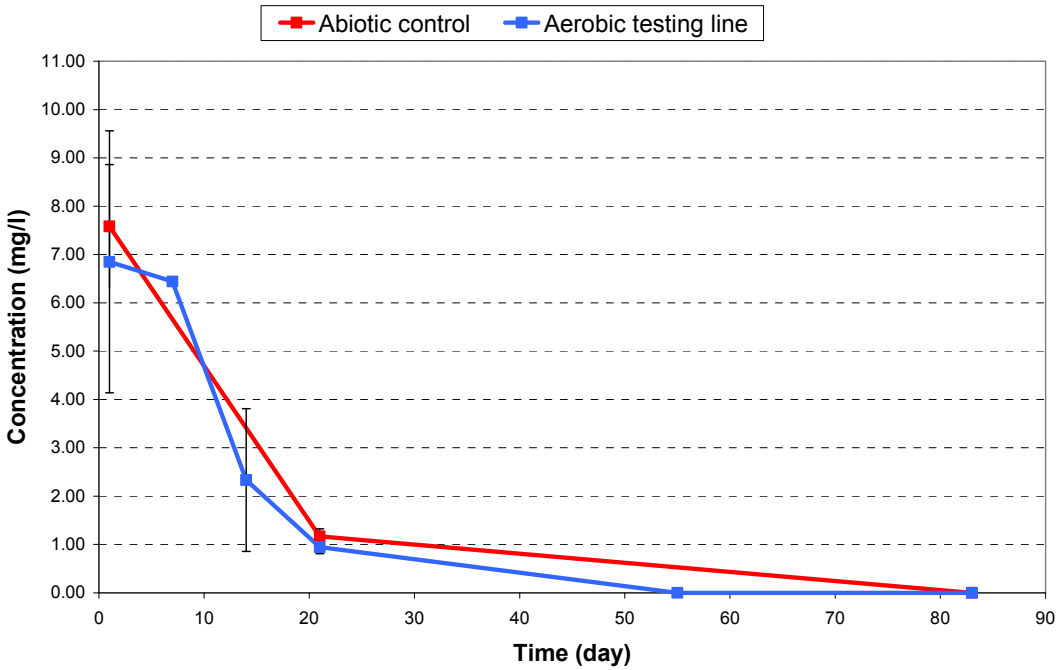


Figure 4.30 – Acenaphthene concentration trend in liquid phase; aerobic experiment

An additional work has been carried out, as in case of anaerobic experiment, to valuate the phenomenon of PAH washing out connected to the use of distilled water to wash the sand out of the flask. In table 4.17 the results are summarized; the terms to read the table are the same explained in the anaerobic experiment.

Instead of anaerobic test, for the aerobic experiment a higher amount of water has been used to wash the flask, since the smaller particles sizes have been formed, resulting in more difficulty to take out from the bottle. In this case, the higher is the amount of water used, the higher is the PAH washing out, with an excess of more than 50%.

For the abiotic control the phenomenon is as relevant as in the aerobic conditions testing line, with an excess percentage equal to 62%.

Day	Mineral medium phase (mg/l)	Water phase (mg/l)	Washing water (g)	Excess (%)
Aerobic conditions				
55	90.37 (8.67)	70.68 (1.72)	44.93 (4.84)	43.9
83	14.76 (7.34)	38.29 (13.69)	59.91 (11.63)	72.2
Abiotic control				
83	4.83 (1.64)	7.84 (1.36)	53.38 (16.60)	61.9

Standard deviation in brackets

Table 4.17 – Concentration in mg/l of 5 PAHs sum in mineral medium and water phase; aerobic experiment, day 55 and day 83

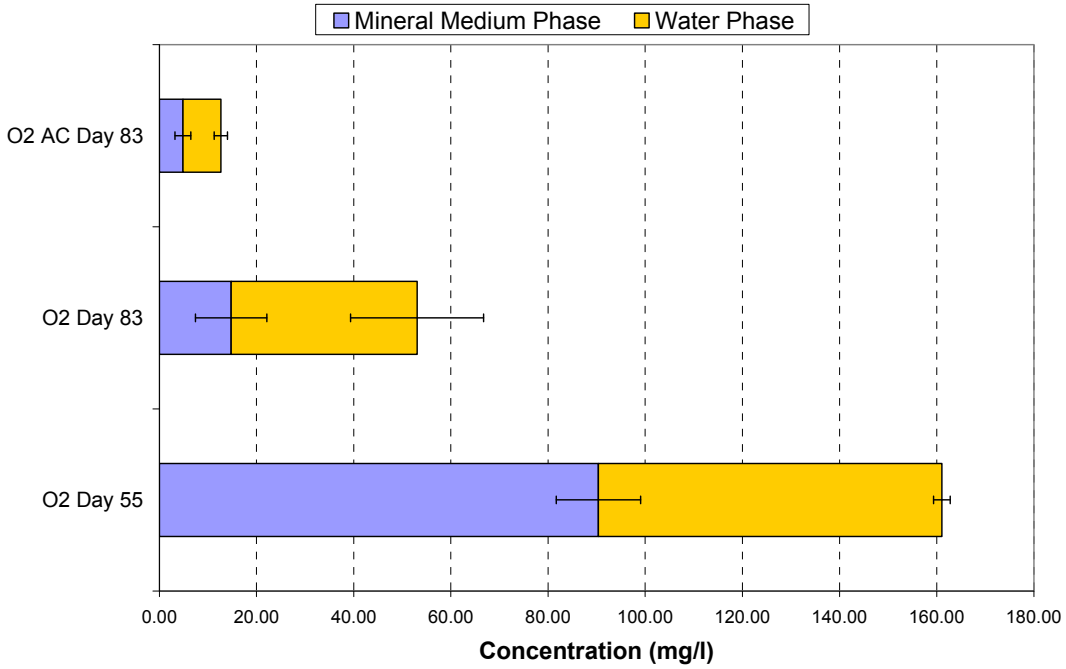


Figure 4.31 – Concentration in mg/l of 5 PAHs sum in mineral medium and water phase; aerobic experiment

4.3.3 Chemical parameters trend

Concerning chemical parameters in the aerobic experiment, in this paragraph pH and dissolved organic carbon will be shown. In the last sampling day, also oxygen concentration has been monitored, to check that anaerobic conditions occurred, and the value detected has been 8.10 mg/l (SD = 0.16), through the oxygen electrodes.
The other parameters are reported subsequently.

4.3.3.1 pH trend

The pH trend over the incubation time has been constant for the first 3 weeks, close to neutrality. In the last sampling day an increase to a basic value of 9.5 has been detected. Values are displayed in figure 4.32.

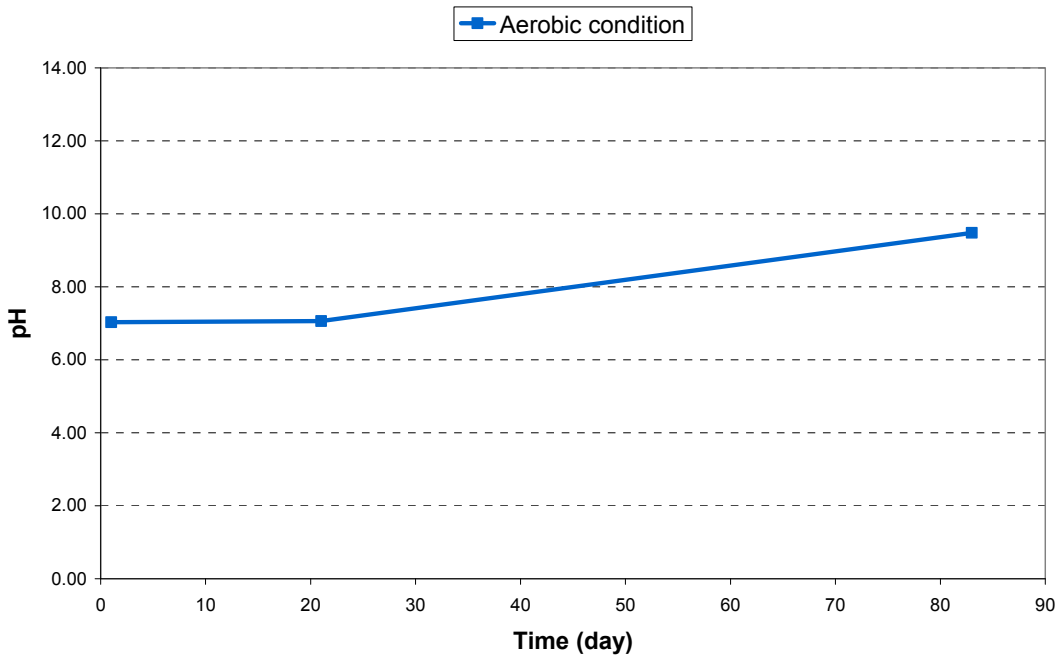


Figure 4.32 – pH trend over the incubation time; aerobic experiment

4.3.3.2 Dissolved organic carbon trend

The dissolved organic carbon measured over the incubation time remained constant and equal to circa 36 mg/l. This is the expected behaviour of carbon in liquid phase, since it should be constant over the whole experiment length or till the complete depletion of contaminants. However the detected concentration of organic carbon in

mineral medium is higher than the expected value, based only on theoretical PAH chemical properties. Results are shown in figure 4.33.

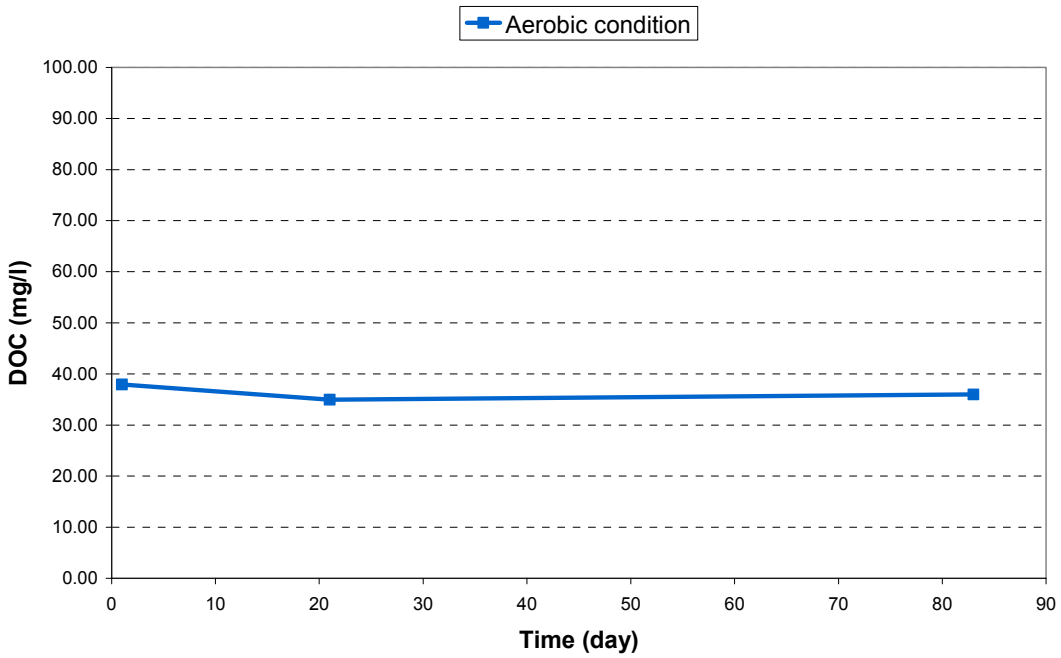


Figure 4.33 – Dissolved organic carbon (DOC) trend over the incubation time; aerobic experiment

Chapter 5
Discussion

5.1 Anaerobic degradation experiment

5.1.1 PAH content in sand phase

According to the aim of this thesis work, the degradation results obtained show that, despite the initial will of supplying optimal conditions for anaerobic biodegradation within the microcosms, no PAH biodegradation has been occurred over the incubation time, since relevant decrease in the PAH content of the sand phase have not been detected for both nitrate and sulphate reducing conditions testing lines. Also abiotic leaks have not been detected, in accordance to the fact that the system was closed and physical losses, as contaminant volatilization, were not possible. The abiotic control confirms this result, because the detected PAH concentration does not change over the incubation time.

In case of acenaphthene, a 40 % decrease of initial concentration has been individualized between day 0 and day 1, but it must be attributed to a physical volatilization of this compound during the sample preparation phase (e.g. filtration) and not to physical or biological removing phenomena occurred during the experiment. This can be justify by the low molecular weight of acenaphthene, which was the PAH with the lowest molecular weight used to spike the sand, exposing it to volatilization phenomena more than other compounds.

The absence of contaminant removal under anaerobic conditions can be attributed to the small amount of microorganisms put inside the system. This is directly connected to the failed achievement of optimal conditions i.e. for oxidation-reduction potential in both nitrate and sulphate testing lines, since microbial PAH degradation is strongly affected by the redox environment (Mihelcic and Luthy, 1988). In fact as reported in paragraph 1.3.4.5, negatives ORP values must be reached to assure optimal conditions for anaerobic biodegradation.

5.1.1.1 Low load of microorganisms

The main explanation of absence of biodegradation in the experiment may be found in the amount and the characteristics of microorganisms. Coates et al. (1997) reported that pre-exposure to PAHs is often necessary to obtain a PAH biological degradation, especially for sulphidogens (Coates et al. 1996), suggesting that low numbers of appropriate microorganisms rather than adverse environment conditions limit PAH content decrease.

Concerning these results, in the anaerobic experiment it is probable that a low load of microorganisms has been put inside the microcosm, for both nitrate and sulphate reducing conditions testing lines. The microorganisms were known to be able to degrade PAH under anaerobic conditions, but probably they required time to adapt their metabolism to the created microcosm. To give them this adaptation time alternative electron donors should have been added to the system, letting the present bacteria to develop a specific bacterial population able to degrade the investigated contaminants.

To support this thesis, most of literature examples concerning PAH biological reduction are carried out with soils coming from contaminated areas, involving a previous exposure to the contamination (Coates et al. 1997, Yuan and Chan 2006, Mihelcic and Luthy 1988, Chang et al. 2008).

5.1.1.2 Oxidation reduction potential conditions

In case of nitrate reducers, ORP reached negatives values in day 83, only after the sludge input, occurred in day 75, going back to positive values in the last two sampling days (see paragraph 4.2.3.1). It is accepted that nitrate reducing biodegradation conditions may occur for values within 50 and – 50 mV, so they have been reached only for a small period over the incubation time.

It was expected to obtain anaerobes conditions and proper ORP values only depriving the microcosm of the oxygen. Although oxygen content measured was very low appropriate redox potential was not achieved. Obviously redox potential depends not only from oxygen content.

In case of sulphate conditions testing lines, appropriate conditions of oxidation reduction potential (about -200mV, see paragraph 1.3.4.5) are reached only after the

inoculation, even though an immediate increase has been detected in the further sampling days. As for nitrate conditions, no biodegradation is detected over the incubation time. In this case this conclusion is supported by the absence in sulphate depletion and in sulphite or sulphide production.
A possible explanation of such high values of oxidation-reduction potential may be directly connected to the low amount of microorganisms put inside the system, as mentioned above (paragraph 5.1.1.1), which did not allow to develop optimal conditions for PAH biodegradation.

5.1.1.3 Working temperature

As said in paragraph 1.3.4.1, temperature is an important parameter affecting the biodegradation. To gain the initial goal of providing good conditions for field application of anaerobic degradation, an average temperature has been chosen varying from 20 to 23°C. By the way most of literature examples, reporting anaerobic PAH degradation, show a higher working temperature unrealistic for natural soil conditions.
Uribe-Jongbloed and Bishop, who carried out a similar experiment using pumice as soil and spiking it with 4 PAHs, demonstrated PAH biodegradation, although they stored the samples inside a temperature-controlled chamber, keeping them at 36°C. Coates et al. (1997) carried out sediment incubation at 25° in the dark, instead Yuan and Chang (2005) incubated the samples at 30°C also in the darkness.
Due to the collected data, the temperature may be included in the factors affecting the absence of microorganisms activity inside the microcosms. But such investigations are far away from real environment conditions.

5.1.1.4 Nitrate decrease

Concerning the nitrate decrease detected during the incubation time, it may be attributed to a mild biodegradation activity of the inocula, that is supported by the production of nitrite; however the carbon source used by bacteria has not been the contaminants. A possible explanation can derive by the fact that after inoculation, carbon is available from the inocula itself, especially in case of added sludge.

5.1.2 PAH content in liquid phase

Very high concentration of PAH has been detected over the incubation time in the liquid phase and the PAH content derived from the water solubility of each compound have not been held by. A possible explanation of such a high concentration may be attributed to the fact that, during the filtration phase, polycyclic aromatic hydrocarbons bounded to the smaller sand particles can go through the filter, affecting the final PAH content in the liquid phase.

In addition results from day 83 and 111 pointed out that the distilled water used to take the sand out of the flask caused a wash-out of PAH compounds, since PAH have been detected also in this water phase. In this way it has been demonstrated that the detected concentrations of polycyclic compounds may be caused also to physical phenomena occurring during the extraction procedure.

The abiotic control, however, has been detected on average with the lowest PAH content in liquid phase, suggesting that the presence of mercury chloride used to poison the samples reduces the solubility of PAH in liquid phase.

5.2 Aerobic degradation experiment

5.2.1 PAH content in sand phase

The aerobic experiment has been carried out, as reported in chapter 2, to permit a comparison between the anaerobic conditions, which are more difficult to manage and less esoergonic, involving a lower growth rate, and the aerobic conditions, which are known to be easier to keep under control and implicates more energetic processes and therefore a faster degradation process.

Unfortunately, as for the anaerobic experiment, no biological activity by microorganisms has been registered over the incubation time. A decrease in contaminants concentration has been detected, particularly concerning acenaphthene content, but the removal must be entirely attributed to abiotic process, such as volatilazation, and not to biological activity. In 21 days a decrease of 80% has been detected, and on day 55 acenaphthene concentration was set to non detectable values. For all the other 4 polycyclic aromatic hydrocarbons no relevant variations has been displayed over the incubation time, involving no bacterial activity.

5.2.1.1 Absence of microbial PAH removal activity

Similar conclusions to the anaerobic experiment may be drawn for the aerobic one. Also in this case the amount of bacteria put inside the system could be too low to permit the growth of specific microorganisms population able to degrade the contaminant substrate.

A different solution may be the addition of alternative electron donors, such as acetate or lactate, to allow the development of a higher bacteria amount and therefore to a specific PAH removal microbial population.

Other explanations, concerning physical parameters as oxidation reduction potential for anaerobic experiment, can not be provided in this case. In presence of oxygen in the water the ORP is always set to proper values to favourite the biodegradation. In addition the reported pH has been detected to be close to neutrality, meaning proper pH conditions for microbial activity. In addition, spherical glasses have been used to allow better oxygen supply to the microorganisms.

5.2.2 PAH content in liquid phase

The PAH content in the liquid phase, similarly to the anaerobic experiment, has been detected higher than the expected value, calculated considering the single water solubility of each compound, over the incubation time. Compared to the anaerobic experiment, the aerobic one displayed higher concentration in liquid phase.

A possible explanation may be found by the fact that organic polycyclic compounds bounded to smaller sand particles can pass through the filter used to separate the sand phase from the liquid one. Moreover the addition of spherical glasses inside the samples and the further breakage of sand particles may cause an increase in the amount of fine size of sand and, therefore, in the PAH content in liquid phase.

The different analysis carried out on day 55 and day 83 pointed out that, similarly to the anaerobic experiment, also the extraction procedure affects the PAH content in liquid phase. Moreover a higher PAH excess has been detected compared to the anaerobic experiment because more water has been used to wash the sand out of the flask.

5.3 Conclusion

Despite the initial aim of investigating optimal conditions for biological anaerobic biodegradation under two different electron acceptors conditions, no PAH removal has been detected over the incubation time for both biotic testing lines. Principal causes of biological activity absence may be attributed to the low bacteria amount put inside the microcosm and therefore to the problems to reach optimal environmental conditions, especially regarding the oxidation-reduction potential, which has been shown to be a fundamental parameter to favour particular anaerobic degradation conditions.

Moreover an aerobic experiment has been carried out, at the same time, to compare a process in the presence of oxygen, considered more energetic and, therefore, able to guarantee a faster degradation, with an anoxic process, considered more delicate and difficult to manage. Unfortunately, also under aerobic condition no PAH removal has been displayed by bacteria.

Comparing the two experiments, abiotic phenomena removal has been observed over the incubation time for the aerobic testing line, particularly referred to volatilization of acenaphthene, suggesting that abiotic phenomena are less marked in anaerobic processes than in aerobic ones and increasing the contaminants removing potential of aerobic biodegradation compared to anaerobic one.

The results of the present study point out the complexity of managing biological process connected to anaerobic PAH removing events in laboratory scale, particularly in creating optimal microcosm properties which are able to favour the microbial activity.

For the further research of PAH anaerobic degradation processes in sandy soils investigation of influence of alternative electron donors on the reaching optimal degradation conditions is suggested.

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