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NITRIFYING AND DENITRIFYING BACTERIAL COMMUNITIES IN THE SEDIMENT AND RHIZOSPHERE OF A FREE WATER SURFACE CONSTRUCTED WETLAND

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Memòria redactada per Olaya Ruiz Rueda, inscrita al Programa de Doctorat en Ciències: Química i Física de les Molècules i els Materials, Biotecnologia i Ciències de la Salut de la Universitat de Girona, per adquirir el grau de Doctor Europeu per la Universitat de Girona

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Vist-i-plau

El director de la tesi — Autora

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SUMMARY

The continuous delivery of nutrients, mainly phosphate and nitrogen, is the major cause of eutrophication of aquatic environments. Treatment technologies based on constructed wetlands have been applied to reduce the levels of nitrogen as a cost-effective alternative compared to conventional treatment methods. The nitrogen removal efficiency in wetlands relies on the presence of plants and the alternation of aerobic and anaerobic conditions to promote both nitrification and denitrification. Although the role of emergent macrophytes in such systems is largely recognized, their contribution to the overall treatment process has not been quantified very frequently. We have investigated the microbial nitrification and denitrification activities in relation to two plant species in a free water surface constructed wetland (FWS-CW), designed to minimize the impact of nutrient release into the Natural Reserve of *Els Aiguamolls de l'Empordà* (Girona, Spain).

Our objective was to evaluate some of the critical factors regulating the distribution and function of ammonia oxidizing bacteria (AOB) and denitrifying microbial populations. The real nitrogen removal efficiencies in the constructed wetland were analyzed with a special emphasis put on the role of vegetation.

The Empuriabrava FWS-CW is subjected to strong seasonal fluctuations or man-derived actions, which imply considerable variations in hydraulic loads and chemistry of the water along the year. From winter situations of typically high water retention times and low nitrogen loads, to the considerable increases in both the volume of water discharged and the relative abundance of ammonia in the influent during summer; the FWS-CW often face extreme situations including the complete drainage of water or partial sediment removal.

The presence of emergent vegetation creates a series of microhabitats that contribute to further increase the spatial heterogeneity within the CW. In addition, the vegetation coverage defines completely different conditions in the three separate treatment cells of the Empuriabrava FWS-CW.

To assess the effects of such a complex range of environmental variations on N transforming communities we employed potential activity experiments, molecular techniques for the microbial community structure analysis and estimations of *in situ* nitrogen removal rates.

The comparison of potential ammonia and nitrate conversion rates between bare sediments and cattail (*Typha* sp.) or reed (*Phragmites australis*) rhizospheres demonstrated the importance of vegetation to stimulate higher nitrogen removal yields. Moreover, during episodes of high nutrient and water loads, activity rates were significantly higher for reed than for cattail, reflecting a better suitability of the first plant species to enhance the treatment performance.

Communities of AOB exhibited a homogeneous profiling that did not vary neither in time or space, but the *nir*S T-RFLP and *nos*Z DGGE patterns indicated shifts in the denitrifying communities coupled to plant species and most notably to wetland hydrology. The seasonal shifts in the community structure were not linked to a variation of the potential denitrification rates for a given plant species, except for events of critical load, when the differences between the denitrifying bacteria associated with *Typha* sp. and *Phragmites australis* roots were more evident.

Our results show that plants are crucial for supporting and controlling the nitrification and denitrification processes in the wetland sediment. However, the most significant changes in

community composition for denitrifiers were driven by environmental factors related with wetland operation and not by plant specific effects. Furthermore, during situations of higher loadings, a greater nitrogen removal activity was inferred for *Phragmites australis* rhizospheres, which was further supported by removal yields estimated *in situ*. The findings open up an interesting topic for future wetland management and design, by considering plant coverage rearrangements as a tool to compartmentalize physiological functions and modulate the nitrogen removal in wetlands

RESUM

La contínua descàrrega de nutrients, sobretot fosfats i nitrogen, és la major causa d'eutrofització dels ecosistemes aquàtics. Els sistemes de tractament basats en aiguamolls construïts s'han emprat per reduir ells nivells de nitrogen a l'aigua com a alternativa de baix cost als mètodes de depuració convencionals. L'eliminació del nitrogen a aquests sistemes depèn en bona part de la vegetació, i l'alternança de condicions aeròbiques i anaeròbiques per promoure els processos de nitrificació i desnitrificació. Tot i que el paper de la vegetació en el tractament es troba àmpliament reconegut, la seva contribució al procés no s'ha quantificat de manera freqüent. En aquest treball hem volgut investigar les activitats microbianes de nitrificació i desnitrificació en relació a dues espècies de plantes macròfites en un sistema d'aiguamolls de tractament de flux superficial (FS-SAC), dissenyat per minimitzar l'impacte de l'alliberament d'aigua carregada de nutrients a la reserva natural dels Aiguamolls de l'Empordà. (Girona, Espanya).

El nostre objectiu fou avaluar alguns dels factors que regulen la distribució i funció de les poblacions de bacteris oxidadors d'amoni (AOB) i desnitrificants al SAC. L'eficiència d'eliminació de nitrogen al SAC es va analitzar posant especial èmfasi en el paper de la vegetació.

El SAC Empuriabrava està marcat per fortes variacions, tant estacionals, com derivades del control operacional del SAC, que causen modificacions considerables del cabal i la composició química de l'aigua. Des de les situacions d'hivern, caracteritzades per elevats temps de retenció hidràulica i baixa concentració de nutrients, fins al considerable augment tant de la càrrega d'aigua com de la concentració relativa d'amoni a l'influent que té lloc durant l'estiu, els aiguamolls poden veure's exposats a situacions extremes, com el drenatge total de les cel.les o la remoció parcial de sediment.

La presència de macròfits crea diversos microhabitats que contribueixen a augmentar encara més l'heterogeneïtat espacial dins els SAC. A més, la particular distribució de les masses de vegetació defineix condicions completament diferents a les tres cel.les de tractament del sistema d'Empuriabrava.

Amb la finalitat de determinar els efectes d'aquest conjunt de variacions ambientals en les comunitats de microorganismes transformadors de nitrogen es van utilitzar mesures d'activitat potencial, tècniques moleculars per l'anàlisi de l'estructura de les comunitats microbianes, i estimacions de les taxes d'eliminació de nitrogen in situ.

La comparació de les taxes potencials de conversió d'amoni i nitrat entre el sediment i la rizosfera de canyís (*Phragmites asutralis*) i balca (*Typha* sp.) va posar de manifest la importància de la vegetació per estimular un major rendiment en l'eliminació de nitrogen.

A més, durant episodis crítics d'elevada càrrega de nutrients a l'influent es van obtenir taxes significativament superiors per canyís que per balca, indicant la millor adequació de la primera espècie vegetal pel tractament.

En referència a la composició de les comunitats microbianes, mentre que els patrons d'AOB no van mostrar variacions respecte cap dels factors considerats, els patrons de T-RFLP del gen *nir*S i DGGE del gen *nos*Z van revelar canvis en les comunitats desnitrificants en relació a la vegetació, i sobretot degut a la hidrologia del SAC.

No obstant, aquests canvis no es relacionaren amb variacions en les taxes d'activitat per un determinat tipus de planta, si bé durant els períodes de càrrega crítica es van pronunciar les diferències existents entre les poblacions de bacteris desnitrificants associats a balca i canyís.
Els resultats demostren que les plantes són un factor crucial per sostenir i controlar les funcions de nitrificació i desnitrificació al sediment del SAC. De tota manera els canvis més significatius en la composició de la comunitat van tenir lloc en relació amb factors ambientals lligats a la operació del SAC. A més, durant situacions de màxima descàrrega es va poder inferir una major capacitat d' eliminació de nitrogen pel canyís, fet que concorda amb les dades dels rendiments estimats *in situ*.
Aquests fets presenten una interessant aplicació pel futur disseny i gestió de SAC de flux superficial, plantejant la reorganització de la cobertura vegetal com a eina per compartimentalitzar funcions fisiològiques i modular l'eliminació de nitrogen en sistemes d'aquest tipus.

RESUMEN

La contínua descarga de nutrientes, principalmente fosfatos y nitrógeno, es la mayor causa de eutrofización de los ambientes acuáticos. Los sistemas de tratamiento basados en humedales de tratamiento representan una alternativa de bajo coste a los métodos convencionales de depuración para obtener la reducción de los niveles de nitrógeno del agua. La eficiencia en la eliminación de nitrógeno en estos sistemas depende de la vegetación, así como la alternancia de condiciones aerobias y anaerobias para promover los procesos de nitrificación y desnitrificación. Aunque el papel que desempeña la vegetación se halla ampliamente reconocido, su contribución al proceso de tratamiento no se ha cuantificado de modo frecuente. En este trabajo hemos investigado las actividades microbianas de nitrificación y desnitrificación en relación a dos especies de plantas macrófitas en un sistema de humedales de flujo superficial (FS-SHT), diseñado para minimizar el impacto del vertido de nutrientes en la reserva natural de los *Aiguamolls de l'Empordà* (Girona, España).

Nuestro objetivo fue evaluar algunos de los factores que regulan la distribución y función de las poblaciones de bacterias oxidadoras de amonio (AOB) y desnitrificantes en el SHT. La eficiencia de eliminación de nitrógeno se analizó en el SHT, poniendo especial énfasis en el papel de la vegetación.

Los humedales de Empuriabrava están sujetos a fuertes variaciones, tanto estacionales, cómo derivadas de las acciones de control en el proceso de depuración, que conllevan modificaciones considerables en el flujo recibido y la composición química del agua a lo largo del año. Desde las situaciones de invierno, caracterizadas por elevados tiempos de residencia hidráulica y bajas concentraciones de nitrógeno, hasta el considerable aumento tanto en la carga de agua como en la concentración relativa de amonio en el influente que tiene lugar durante el verano, los humedales pueden además padecer situaciones extremas como el completo drenaje de las celdas o la retirada de sedimento.

El establecimiento de plantas macrófitas crea diversos microhabitats que contribuyen aún más a aumentar la heterogeneidad espacial en el SHT. Además, la particular distribución de las masas de vegetación define condiciones completamente distintas en las tres celdas de tratamiento de los humedales de Empuriabrava.

Con tal de determinar los efectos de tal conjunto de variaciones ambientales en las comunidades transformadoras de nitrógeno se emplearon experimentos de actividad potencial, técnicas moleculares para el análisis estructural de las comunidades microbianas, y estimaciones de las tasas de eliminación de nitrógeno *in situ*.

La comparación de las tasas potenciales de conversión de amonio y nitrato entre el sedimento y la rizosfera de carrizo (*Phragmites australis*) y espadaña (*Typha* sp.) puso de manifiesto la importancia de la vegetación para estimular un mayor rendimiento en la eliminación de nitrógeno.

Además, durante los episodios críticos de alta carga de nutrientes en el influente se obtuvieron tasas significativamente superiores en las muestras de carrizo que en las de espadaña, indicando su mayor adecuación para el tratamiento.

En referencia a la composición de las comunidades microbianas implicadas, mientras los patrones de las comunidades de AOB no mostraron variaciones respecto a ninguno de los factores considerados, los patrones de T-RFLP del gen *nir*S y DGGE del gen *nos*Z revelaron cambios en las

comunidades desnitrificantes asociados a la vegetación, aunque más notablemente debido a la hidrología del sistema.

Estos cambios sin embargo no derivaron en variaciones de las tasas de actividad para un tipo de planta, si bien en los períodos de alto vertido de nutrientes se pronunciaron las diferencias existentes entre las poblaciones de bacterias desnitrificantes asociadas a espadaña o carrizo.

En resumen, en este trabajo se pudo demostrar que las plantas son un elemento crucial para sostener y controlar la nitrificación y la desnitrificación en el sedimento de los humedales. De todos modos, los cambios más significativos en la composición de las comunidades desnitrificantes ocurrieron en relación a factores ambientales ligados principalmente a la operación del SHT. Durante situaciones de máxima carga, se pudo inferir una mayor capacidad de eliminación de nitrógeno en la rizosfera de carrizo, hecho que concuerda con los datos de rendimiento estimado *in situ*.

Estos hechos abren un interesante tema para el futuro diseño y gestión de SHT de flujo superficial, planteando la reorganización de la cobertura vegetal como herramienta para la compartimentalizar distintas funciones fisiológicas y modular la eliminación de nitrógeno en sistemas de este tipo.

CONTENTS

1

SCIENTIFIC BACKGROUND

Wetlands are the transitional link between water, aquatic ecosystems, and land, terrestrial ecosystems. They include marshes, swamps and bogs found along the edges of rivers, ponds, lakes and coastal lines and constitute habitats of unique and exceptional ecological value from many points of view. Due to their particular features, wetlands act as biodiversity reservoirs, fostering countless species of characteristic flora and fauna. Wetlands assume a critical and irreplaceable role in the global water cycle, as they provide flood control, storage of storm and runoff waters and water purification, as well as participating in shoreline stabilization. The beneficial functions of wetlands also include sediment and nutrient retention and export functions, and recreational and cultural opportunities (ramsar.org).

Wetlands occur everywhere on Earth, from the tundra to the tropics. The UNEP-World Conservation Monitoring Centre has suggested an estimate of about 570 million hectares, roughly 6% of the Earth's land surface, of which 2% are lakes, 30% bogs, 26% fens, 20% swamps, and 15% floodplains. Mitsch and Gosselink, in their standard textbook Wetlands (80), estimate that 4 to 6% of the Earth's land surface is covered by these particular habitats of transition. Mangroves cover

some 240,000 km^2 of coastal area. Nevertheless, a global review of wetland resources prepared by Ramsar COP7 in 1999, while affirming that "…it is not possible to provide an acceptable figure of the areal extent of wetlands at a global scale…", indicated a 'best' minimum global estimate of between 748 and 778 million hectares, which may be increased to 4,462 million ha depending on the data sources.

1.1. Constructed Wetlands

The intensification of industrial and agricultural practices, together with the continuous development of urban areas, has caused a substantial increase in nutrient discharges to the environment. This has led to eutrophication and the loss of the ecological quality of many natural water resources, as the most pressing environmental problems.

Conventional water treatment technologies are applied routinely to ensure minimum standards of water quality before being discharged to the environment (European Directive 91/271/CEE). These systems, usually known as Wastewater Treatment Plants (WWTP), perform satisfactorily but their construction, operation and maintenance entails substantial capital and energy costs, which raises the need for more sustainable alternatives. Treatment strategies based on constructed wetlands (CW) are classified within these cost-effective treatment systems.

Wetlands have traditionally been considered as water polishing systems (59, 125), but over the past two decades they have gained a lot of popularity and have become widely implemented (18, 46) in urban settlements as engineered water treatment ecosystems. They can be applied to unconventional on-site treatment of diffuse or non-point polluted water, such as that derived from industrial and rural management, or wastewater from small settlements (46). Apart from treating municipal waste, many countries have preferred to adopt and integrate constructed wetlands into multistage treatment systems for the final polishing of conventional WWTP effluents (123). The main concerns usually associated with CW performance include the closer approximation of nutrient concentrations to adequate standards in order to minimize the impact on recipient waters.

In this sense, recent research has focused on removing nitrogen from municipal wastewater and agricultural drainage by exploiting wetlands technologies (2, 15). Man-made nitrogen discharges from agriculture and industry are one of the main causes of eutrophication of freshwater ecosystems. In addition, an overload of nitrogen can lead to increased emissions of nitrous and

nitric oxides (N_2O, NO), greenhouse and ozone-deteriorating gases, via microbial denitrification. Nitrogen pollution is not only an environmental health hazard (59) but also causes toxicity problems for end users. Negative effects of nitrates in drinking water are especially critical for children under six months due to the development of methemoglobinemia (27).

Constructed wetlands basically mimic the functioning of natural systems, but the processes for improving water quality are regulated and forced to occur at a higher speed. Critical parameters influencing the removal rate of contaminants include the water flow or load, the preferred direction of flow, the hydraulic retention time and the mean depth. All of these are controlled in a way that larger loads of pollutants can be treated in a more predictable manner.

Figure 1. A) Horizontal Sub-Surface Flow Constructed Wetland located in Les Franqueses del Vallés, Catalonia (Spain) (www.ingeniabios.com). B) Picture of a vertical subsurface flow reed bed constructed wetland in Ireland (www.wasteworks.ie).

Briefly, the treatment is carried out by a complex series of physical, chemical and biological processes in which the water, sediments, plants and microbes inhabiting the different compartments interact. The presence of vegetation in CWs guarantees successful performance, since plants exhibit both structural and biochemical properties upon which many of the mechanisms involved in the removal of pollutants rely (18). As a result, the organization of plant masses within the wetlands is generally included as a key component in initial wetland design.

Furthermore, CWs are suitable for the treatment of fluctuating water levels, and thus confer high operation flexibility. Only the large extension of land required for their construction, as well as a

higher susceptibility to climatic conditions, may limit the application of this approach in some geographic areas (58).

1.1.1. Regime flow alternatives

Constructed wetlands admit variations in the construction criteria and may be operated differently according to several specific designs. These configurations differ in the degree of efficiency with which some of the processes involved in water treatment are carried out. Their applicability is therefore dependent on the specific treatment functions to be attained.

Table 1. Summary of the characteristics of different constructed wetland types. Adapted from Kadlec *et al*. (1996) (58)

	Advantages	*Disadvantages*
H-SSF		
	• Greater treatment surface • High organic consumption rates • Decreased odour production • Decreased insect proliferation • Higher tolerance to climatic conditions	• Limited aeration • Poor potential for nitrification • Large extension of land required
V-SSF		
	• Minimized treatment area than H-SSF • Better oxygen transfer, higher nitrification than H-SSF • Suited to small communities where inexpensive land and low cost media are readily available. (US EPA, 2000)	• Higher construction costs, restrict large spatial development • Less efficient in removal of suspended solids than H-SSF • Often need further polishing in H-SSF
FWS		
	• Combine aerobic and anaerobic conditions • More efficient nitrogen removal than SSF • More suitable in warmer climates • Well suited for small communities • Aesthetic appeal, recreational and environmental education activities	• Large extension of land required for construction • Higher susceptibility to climatic conditions • Potential exposure of water to human contact

Primarily, two flow regime alternatives are possible, namely subsurface flow (SSF-CW) and free water surface (FWS-CW) systems. SSF-CWs confine water flow through a soil medium without direct exposure to the atmosphere, while FWS-CWs typically emulate natural wetlands and consist

of a water layer of different depths flowing freely over an impervious artificial substrate bed. In vertical subsurface flow constructed wetlands (VSF-CW), wastewater is loaded onto the surface of a planted filter bed and forced vertically through the sediment. In VSF-CWs the pollutants are primarily removed by microorganisms growing over the sediment particles or plant roots. The three set-ups perform differently and have particular characteristics and applications which are summarized in Table 1 (35, 58).

Table 2. Description of principal contaminant removal and transformation mechanisms in free water surface (FWS) and vegetated submerged beds (SSF). Adapted from Crites and Tchobanoglous, (1998) (28)**.**

Contaminant	FWS-CW system	SSF-CW system
Organic Material	Bioconversion by aerobic facultative, and anaerobic bacteria on plant and debris surfaces (soluble BOD) Adsorption, filtration, and sedimentation (particulate BOD)	Bioconversion by facultative and anaerobic bacteria on plant and media surfaces Adsorption, filtration, and sedimentation (particulate BOD)
Trace organics	Volatilization, adsorption, photolysis, biodegradation	Adsorption, biodegradation
Suspended solids	Sedimentation, filtration	Filtration, sedimentation
Nitrogen	Nitrification/denitrification, microbial/plant uptake, volatilization.	Nitrification/dentrification, uptake, volatilization
Phosporous	Sedimentation, soil sorption, plant and microbial uptake	Filtration, sedimentation, media sorption, uptake
Heavy metals	Adsorption of plant and debris surfaces, sedimentation, plant uptake	Adsorption to media surfaces, sedimentation, plant uptake
Pathogens	Natural decay, predation, UV irradiation, sedimentation	Natural decay, sedimentation, predation

Removing pollutants in FWS-CWs implies the interaction of a complex series of biochemical and hydrological processes. These processes are largely dependent on factors such as hydraulic retention time and temperature and include physical sedimentation, filtration, and chemical as well as biological transformations (Table 2). The considerable effect of physical conditions on FWS-CW performance determines its degree of application in different regions or climates. Generally,

the water flow controls the oxygen, carbon and nitrogen availability and exchange at the sediment-water interface, which affects the metabolic processes leading to the expected treatment functions.

1.1.2. Role of Vegetation in Surface Flow Constructed Wetlands

The presence of vegetation in FWS-CWs has been associated with increased final effluent quality and higher nutrient removal rates (6, 73, 113). The initial design of a CW for wastewater treatment generally involves defining a spatial distribution pattern of the vegetation according to different parameters. Plant communities are fundamental for ensuring that many processes involved in removing pollutants are effectively carried out by both direct and indirect effects (44). On one hand, vegetation largely affects the hydrology by controlling the path the water preferentially follows, slowing down the water flow velocity and promoting the sedimentation of particles. On the other hand, plant submerged structures act as physical filters and provide surfaces for the establishment of microbiota. In addition, rhizomes and roots of macrophytes also participate in soil stabilization, reduce the impact of the wind on the water column and minimize the resuspension of solid particles (19, 110, 115).

Far from being limited to the above mentioned structural properties, the contribution of plants to water treatment also involves active metabolic actions that favour microbial activity. They contribute to the production of organic carbon through exudates for heterotrophic metabolism (97), increase oxygen availability in the sediments, thereby enhancing mineralization and nitrification (5, 71, 96), and promote denitrification by pulling nitrates from the water column into anaerobic zones in the sediments through the roots (76). Emergent macrophytes have large structures and extensive rhizome development that contribute well to all these processes. The influence of submerged structures in the water column includes an active oxygenation process, which allows microbiota found in natural wetlands to develop (118).

This diversification of functions opens interesting perspectives in wetland design. For example, structural and hydrological characteristics can be modulated to favour or restrain the development of either emergent or submerged vegetation in certain areas, depending on the polishing functions needed in each situation. This offers the possibility to compartmentalize functions by sequentially placing distinctively vegetated sections, each specialized in a given process, thereby improving water quality (119). Plant species are primarily selected according to their tolerance to climatic

conditions, and the maintenance efforts required for keeping the community balanced. Plants showing active growth and large root development are preferred since they reach high biomass levels and form very dense communities in a shorter time. Several plant species belonging to the genera *Scirpus*, *Eleocharis*, *Cyperus*, *Juncus*, *Phragmites* and *Typha* meet these requirements satisfactorily. Among these species, *Phragmites australis, Typha latifolia* and *Scirpus* spp. are the most commonly found in many European countries, including Spain.

Figure 2. General aspect of cattail *(Typha latifolia*) (right) and reed (*Phragmites australis)* communities in a wetland (http://www.ri.nrcs.usda.gov/news/successstories/Archives/WRP.html).

Despite all the above mentioned characteristics of plants, direct nitrogen assimilation by plant tissues has been shown to contribute poorly to the net N removal in wetlands (113). Plants stimulate N removal in sediments by providing both aerobic conditions for nitrification and organic matter for denitrifying organisms. The presence of emergent macrophytes has been shown to consistently favour higher nitrogen removal rates (118) and usually differences in the efficiency of nitrogen removal can be measured when different plant species are used, being cells planted with *Phragmites* spp. those having better removal capacities (44). Similarly, interspecific and seasonal variations in the nitrification and denitrification capacity have been found, which could be attributed mainly to changes in both the quality and quantity of carbon exudates on the surface of the leaves and roots (61).

It seems clear that vegetation sustains many treatment functions; however, their effects can be reversed and a series of problems in wetland performance may arise when the proper balance between vegetation masses and open water is lost and the plant coverage becomes excessive. Nitrification and organic matter decomposition can become severely restricted by low oxygen concentrations in densely vegetated areas, while the accumulation of plant detritus contributes to internal nutrient loading and can give rise to stagnant areas. Furthermore, other problems can arise from the plants shading the water column, since this limits the photolysis needed for pathogen removal and detoxification of organic compounds. It also reduces the autotrophic algal and microbial nutrient retention capacity (54, 115).

1.2. Nitrogen contamination in the environment

In nature nitrogen exists in numerous oxidation states either as particulates or dissolved ions in organic or inorganic matter. The conversions between these forms are mostly mediated by microorganisms (Figure 3), and make up one of the most complex biogeochemical cycles involving several metabolic reactions, from nitrogen fixation to denitrification, and many different microorganisms, from diazotrophs to nitrifying bacteria.

The major source of nitrogen in the environment is the atmosphere, where it exists as N_2. Diazotrophs are symbiotic or free living prokaryotes able to break the triple covalent bond of dinitrogen gas and assimilate nitrogen into organic matter. Nitrate and ammonia can also be incorporated into amino acids and proteins in living cells by an assimilation process. The aerobic or anaerobic decomposition of this organic matter is called mineralization or ammonification and produces the transformation back to ammonia again. Ammonia can be oxidized to nitrate through nitrification, a two-step aerobic process that is critical for the mobility of organic nitrogen in soils and sediments. Two different functional groups of bacteria, ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), catalyze true nitrification. Until now the ability to oxidize ammonia had been thought to be restricted to AOB. However, recent metagenomic studies that revealed the existence and expression of *amo*A genes affiliated with uncultivated, mesophilic *Crenarchaeota* (121) and the recent isolation of an ammonia-oxidizing archaeon (*Nitrosopumilus maritimus*) (67) raised an issue for discussion, since the AOA may have a significant but so far

unrecognized role in the global nitrogen cycle. To date, the AOA have been demonstrated to be ubiquitous in marine and estuarine sediments in oxic and suboxic water columns (43) and

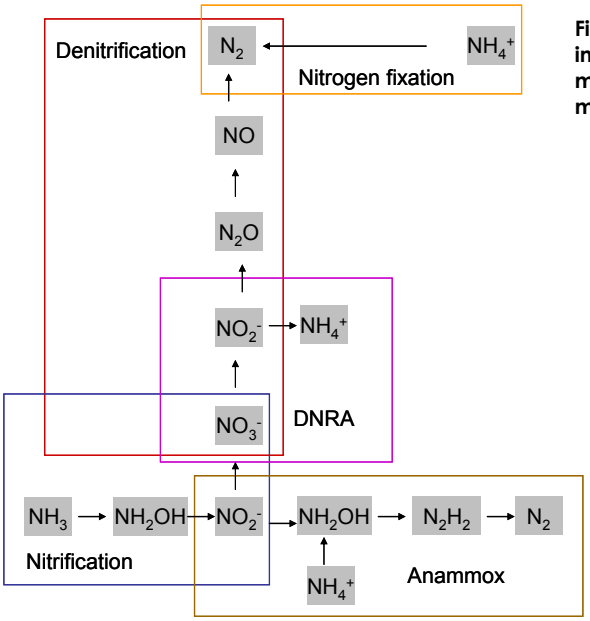

Figure. 3 Main chemical reactions involved in the nitrogen cycle catalyzed by microorganisms. Boxes group common metabolisms, names are indicated.

wastewater reactors (88). In addition, they have been shown to outnumber AOB in soil environments (72). Recent studies point to the relevance of such organisms in the activity in soils (120), although more investigations should be conducted to ascertain whether their contribution to nitrification activity is really significant in other environments.

Next, the nitrate can be reduced through denitrification, defined as a microbial respiratory process during which soluble nitrogen oxides are used as alternative electron acceptors. This consists in the sequential reduction of nitrate into dinitrogen gas and involves several reactions and enzymes. If denitrification is not completed, it can result in the emission of nitrous oxide, a gas that contributes to the greenhouse effect and to the depletion of the ozone layer through stratospheric NO production (29, 53). Despite these negative effects, denitrification is a process of interest in wastewater treatment systems, where it provides for the effective removal of nitrogen from polluted water thereby lowering negative impacts of nitrogen accumulation in aquatic habitats.

Nitrate may also undergo dissimilatory nitrate reduction to ammonia (DNRA), carried out by microorganisms harbouring a specific periplasmic nitrate reductase. This step blocks the final reduction to N_2. Generally, DNRA is favoured when nitrate is limiting and denitrification dominates when there is a limited supply of carbon (26).

Other processes intervene in the nitrogen cycle such as the anaerobic ammonia oxidation to N_2, coupled to using NO_2^- as an electron acceptor (ANAMMOX process) performed by a characteristic group of bacteria (81, 122). Although widely distributed, the significance of the anammox process in soils has yet to be proven though it has been suggested to largely account for nitrogen conversions in marine habitats (31, 70).

Basically, in treatment wetlands designed to remove nitrogen from municipal sewage water that receive an input of mainly ammonia, the pathway to obtain the removal initially involves a nitrification step in the aerobic areas followed by the anaerobic elimination of nitrates by denitrifying bacteria (56, 124).

1.3. Analysis of microbial communities by molecular methods

The inability to classify microorganisms using criteria based on physiological or biochemical features has hampered the study of the diversity of natural microbial communities. Furthermore, the traditional culture dependent methods provide an unreliable representation of the microorganisms present in a sample, since only a small fraction of bacteria is actually estimated to be cultivable (1).

The work of Woese (126) provided tools to establish a classification by comparative sequence analysis. These findings revolutionized the world of microbial ecology and stimulated the development of many molecular biology techniques to explore the structure, function and dynamics of bacterial communities, which are currently applied routinely in this field (Figure 4).

However, the need for culturing is still high, not only to provide better background data for further development of these molecular techniques, but also to understand the link between function and structure (30). Some new isolation approaches, which emulate to a greater extent the environmental conditions from which the samples are taken, have been applied and have resulted

in higher diversity estimates of recovered bacteria (34). Single cell selection methods with further cultivation have been successful for isolating novel AOB (10).

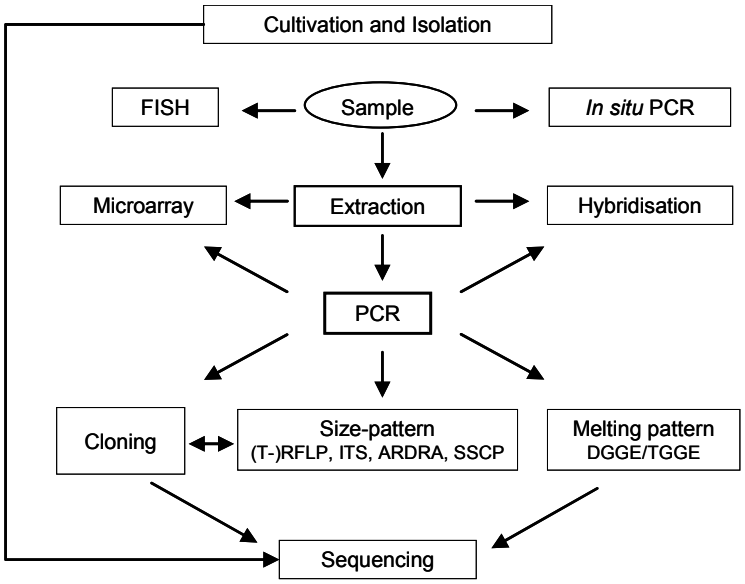

Figure 4. Overview of the most commonly used approaches to explore the diversity of microbial communities in microbial ecology. Arrows indicate possible analysis options. ARDRA, amplified rDNA restriction analysis; DGGE, double-gradient gel electrophoresis; FISH, fluorescent in situ hybridization; ITS, internal transcribed spacer; SSCP, single-stranded conformation polymorphism; TGGE, temperature-gradient gel electrophoresis; (T)-RFLP, terminal restriction fragment length polymorphism. Extracted from Dahllof, 2002 (30).

1.3.1. PCR based methods for community structure analysis

PCR based methods imply extracting nucleic acids and amplifying selected molecular markers further by using specifically designed primer combinations. The structure of microbial communities is finally analyzed by taking advantage of the polymorphism in the mixed pool of sequences by molecular fingerprinting techniques, through cloning and sequencing, hybridization with probes or microarrays or using a combination of these techniques. All PCR based methods are subjected to some limitations, generally related to the nucleic acid extraction protocol used, the inherent biases of the amplification reaction and the efficiency in separating individual markers from a complex and mixed population.

Cloning and sequencing

Cloning and sequencing protocols are considered to be the best exploratory method in molecular ecology as it provides exhaustive and detailed phylogenetic information and allows more accurate diversity estimates, providing a wide screening over a sufficient number of clones. It consists of inserting the various polymorphic sequences contained in a PCR product into vectors and transforming competent cells to stably store the genetic information, which can then be sequenced to reveal the taxonomic affiliation of microorganisms (Figure 5). Nevertheless, cloning experiments are too laborious and time consuming, and thus not appropriate for most experimental studies in which the analysis of multiple samples is required.

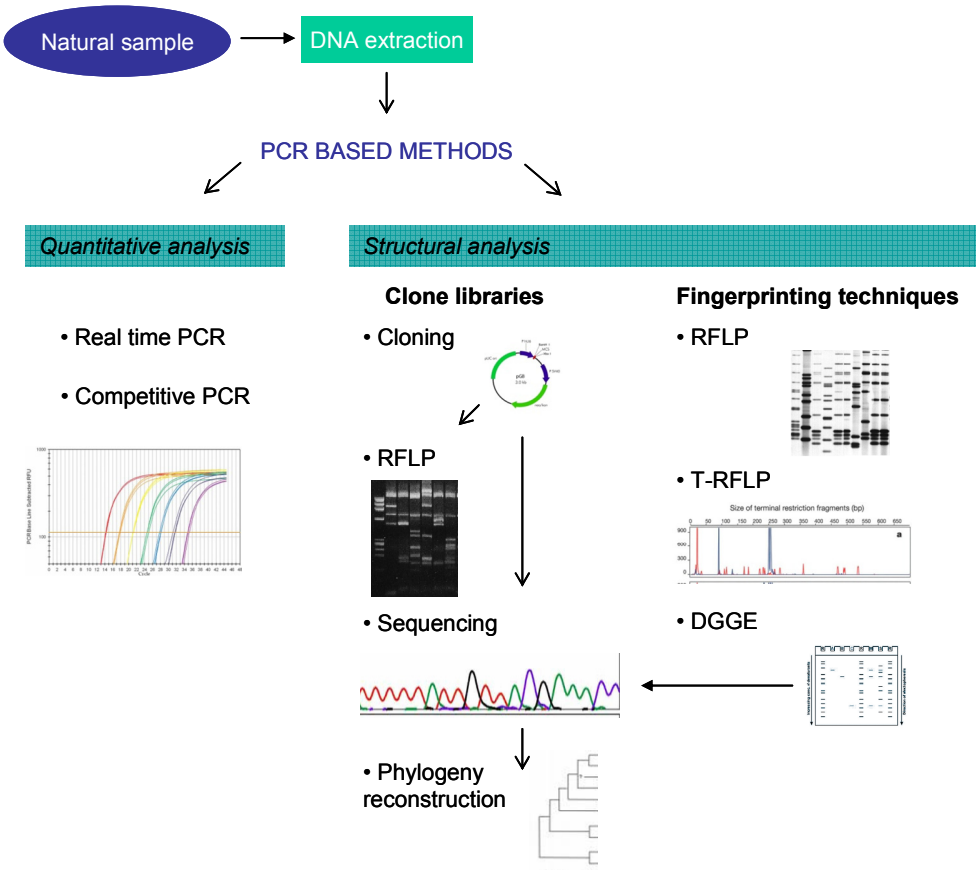

Figure 5. Common PCR based molecular approaches to explore both the structure and density of communities in microbial ecology.

Fingerprinting methods

Genetic fingerprinting techniques were developed to overcome main cloning-sequencing drawbacks and allow a faster analysis of community structure. Therefore, they are best suited to studies of population dynamics, which generally entail the simultaneous analysis of a large number of samples (38). Despite the fact that approaches based on fingerprinting do not offer a real estimation of the genetic diversity within a sample (30, 82), the greater simplicity of such techniques compared to cloning analysis allows changes in microbial communities to be more readily monitored.

Basically, fingerprinting techniques provide a pattern which reflects the structure of microbial communities (82). Some of the methods even allow further identification of members of the community by DNA sequencing. The techniques differ in the basic principle used to obtain the separation of the distinct DNA fragments. Furthermore, they may provide semi quantitative information, and possess variable resolution and sensitivity levels.

There are many environmental studies in which the nitrifying and denitrifying bacterial communities have been investigated by fingerprinting methods such as restriction fragment length polymorphism (RFLP), terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE).

Restriction Fragment Length Polymorphism (RFLP)

The RFLP method is based on the amplified rDNA restriction analysis (ARDRA) technique; however, it uses functional genes as genetic markers. The potential of the method relies on sequence differences in the primary DNA structure of amplified fragments. Different digestion profiles are generated due to variations in the position of selected sequences that act as cutting targets for restriction endonucleases. This requires longer fragments (> 500bp) than DGGE, and cannot be used as a measure of real diversity since the number of bands obtained does not reflect the number of different populations. However, it is a suitable method for screening clone libraries or isolates before DNA sequencing that has been used in numerous studies concerning denitrifying and nitrifying bacterial diversity (13, 25, 40, 48, 51, 74, 91, 103, 105, 111, 128).

Terminal Restriction Fragment Length Polymorphism (T-RFLP)

This method is very similar to RFLP but mixed populations are resolved by a single, usually the terminal, fragment of the digested DNA pool. Either the forward or reverse primers can be labelled with a fluorescent tag that becomes incorporated into the amplified gene fragments during the PCR reaction. After digestion with restriction enzymes, each amplified gene generates a single fragment harbouring a fluorescent end. Ideally, the sizes of terminal fragments are species specific. Labelled fragments can be separated and quantified by automated capillary electrophoresis, which confers a high sensitivity and resolution to the method. The main drawback of the method is that different sequences can result in the same terminal fragment, which makes the correlation of every peak to a defined microbial species difficult, even when multiple restriction endonucleases are used. T-RFLP has been used to explore denitrifying communities for most of the functional markers involved in denitrification and nitrification (3, 11, 14, 16, 23, 52, 79, 127, 129). Extensive work on statistical analysis of similarities has been carried out to interpret the T-RFLP data set and provide reliable measures of relative abundances of bacteria.

Denaturing gradient gel electrophoresis (DGGE)

In this case the principle for separating the various amplicons is based on the fact that variation in the sequence affects the melting behaviour of the secondary structure of DNA. PCR products are loaded in polyacrylamide gels and forced to migrate along a temperature or chemical gradient of increasing denaturing conditions. Each sequence type displays a characteristic migration position after the electrophoresis is complete, rendering a heterogenic mixture of different bands at different positions: a molecular *fingerprint*. DGGE enables, besides mapping shifts in the community structure, further sequencing and phylogenetic characterization of bands by gel excision and purification, followed by sequencing.

Ammonia oxidizing communities have often been investigated by DGGE of ribosomal and functional genes (7, 57, 69, 77, 84). This has also been successfully used for screening and comparing denitrifying communities in environmental samples based on *nir*K and *nos*Z genes (114).

Nevertheless, its analysis is limited to short fragments (ca 500bp), and not exempt from resolution problems that hamper the exact determination of the community complexity. Co-migration of

fragments with different sequences and the appearance of double bands caused by primer degeneration or the formation of heteroduplex molecules are the most commonly documented disadvantages of DGGE. In addition, molecules with multiple melting domains generate profiles rich in fuzzy bands, which make understanding gels difficult. All these reasons stress the need to carefully interpret and extract conclusions regarding the diversity of samples from DGGE fingerprints.

1.3.2. Quantitative PCR based methods

Quantitative methods based on PCR reactions, such as real-time PCR (RT-PCR) or competitive-PCR (cPCR) have also been developed to determine bacterial abundances from the measurement of gene copy numbers. RT-PCR relies on the use of fluorescently labelled selective markers, which allow the continuous quantification of gene copies belonging to a certain group or species during the amplification reaction. The amplification kinetics can be related to the original copy number in the sample by using the appropriate standards. Quantification using cPCR is based on the comparison of the ratio between a target DNA (sample) and a competitor DNA of known concentration (control) in a single PCR reaction. Both methods have been applied satisfactorily to quantify the number of gene copies of *amo*A and several genes involved in the denitrification pathway (36, 49, 50, 72, 75, 78, 95).

Summarizing, molecular approaches do not solve for all biases and limitations of culture-dependent techniques but provide with complementary valuable data for the study of microbial ecology. Molecular methods have been recently considered to be better tools to study microbial ecology than culture dependent techniques providing the higher and unexpected "diversity" of microorganisms that can be retrieved. However, despite they are scarce, comparative studies in which both culture-dependent and independent techniques have been applied on the same sample reveal that most of the phylotypes recovered after cultivation were not detected by molecular fingerprinting (37, 39, 83). Besides, all culture independent methods have intrinsic limitations associated with the principle in which the technique is based.

Not many experimental work is available where a comparison of different molecular methods is made using the same extracted samples to provide with a realistic evaluation of pros and cons of different methods (86, 108). It is generally accepted that cloning and sequencing, although

cumbersome, provide data of good quality but may not be suitable for in depth study of the microbial dynamics in the ecosystem. Fingerprinting techniques, such as those used here, are faster options providing simultaneous and reproducible analyses of multiple samples. T-RFLP offers a very high sensitivity susceptibility and reproducibility and is not subjected to gel-to-gel variations; however, single peaks can contain multiple restriction fragments differing in two or three base pairs that are hardly distinguishable. T-RFLP is the best option for a routine analysis. The main advantage of DGGE is that allows an easier identification of the members of the community through excision and sequencing of differentiated bands, but comparison of samples loaded in different gels may become a difficult task despite the use of internal standards. Both methods have been proven to lead to similar experimental observations and conclusions.

1.3.3. Functional markers for surveys of denitrifiers

Denitrifiers are ubiquitously found and are abundant in soils and sediments (111). They constitute a phylogenetically diverse group, with members identified in more than 60 genera including *Bacteria*, *Archaea* and Fungi (131). The ability to denitrify is then highly dispersed among taxonomical divisions and cannot be associated with specific groups. Interestingly, several denitrifying bacteria are also involved in other steps of the nitrogen cycle, such as nitrification or nitrogen fixation (32, 106, 121).

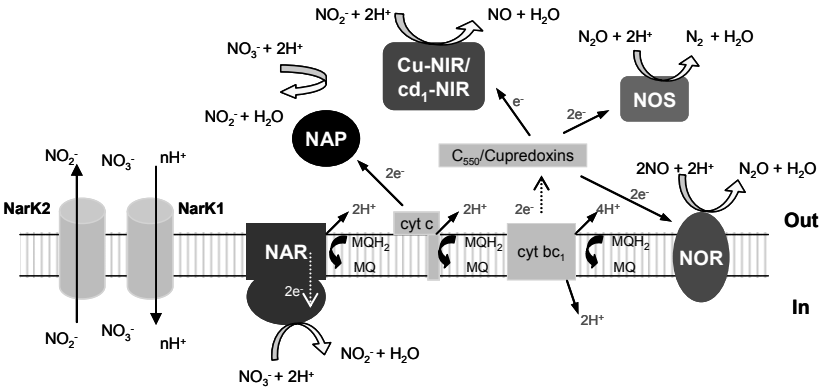

Figure 6. The denitrification pathway, showing the different genes encoding the enzymes involved in each reduction step. The periplasmic nitrate reductase (NAP), the membrane-bound nitrate reductase (NAR), the periplasmic nitrite reductases (Cu-NIR or cd_1-NIR), the membrane-bound nitric oxide reductase (NOR) and the periplasmic nitrous oxide reductase (NOS) are schematically represented. Adapted from Cabello *et al*, 2004 (20)

Denitrifiers are commonly heterotrophic organisms, although some chemoautotrophic or phototrophic bacteria have also been described. The diversity of denitrifiers includes some pathogenic bacteria to plants, animals and humans (131).

Table 3. Main characteristics of functional genes used to study denitrifying bacteria. The analysis is restricted to certain groups of bacteria due to the currently available tools and specificity of markers for denitrification. The groups are indicated.

Gene/ *protein*	***Detected bacteria***	***Additional functions***	***Selective for denitrifiers***
• ***nap*A** Periplasmic nitrate reductase	Gram -	Redox E dissipation	No *
• ***nar*G/** Membrane bound nitrate reductase	Gram - / +		No *
• ***nir*S/** Cyt cd1 nitrite reductase	Gram - / +		Yes
• ***nir*K/** Copper nitrite reductase	Gram -		Yes
• ***nor*B/** Nitric oxid reductase		Nitrosative stress protection	No
• q***nor*B/** Quinol nitric oxid reductase		Nitrosative stress protection	No
• ***nos*Z** Nitrous oxide reductase	*Proteobacteriaceae*		Yes

* non-denitrifying nitrate-reducing bacteria

The high distribution of denitrifying bacteria between taxonomic divisions implies their analysis by molecular methods is only possible by focusing on functional markers of the genes involved in the four steps of the nitrate reduction process. However, it should be emphasized that many of these metabolic steps are catalyzed by at least two different enzymes, and that the multiple enzymes that exist can be present in a single microorganism. Together, these facts make it difficult to provide a comprehensive study of the denitrifying communities, which is only possible with a combination of different molecular markers.

The genes encoding the seven enzymes that catalyze the four steps of the denitrification pathway have been described (Figure 6) and several molecular tools for targeting them by PCR have been developed (Table 3, 4) and used to assess the structure of denitrifying communities in a broad collection of environments. The most relevant investigations carried out so far on the diversity of

Table 4. PCR primers used to target *nir*S, *nir*K and *nos*Z genes involved in denitrification. Relative positions refer to the *nir*S gene sequence of *Pseudomonas stutzeri* ZoBell ATCC 14405 (X56813), the *nir*K gene of *Alcaligenes faecalis* S-6 (D13155), and the *nos*Z gene of *Pseudomonas aeruginosa* DSM 50071 (X652779. Extracted from Molecular Analysis of Soil Denitrifying Bacteria, L. Philipott and S. Hallin (90).

Primer	Position	Primer sequence 5'-3'	Ref
***nir*S**			
nirs 1F	763-780	CCT A(C/T)T GGC CGC C(A/G)C A(A/G)T	(12)
F1acd	856-871	TA(C/T) CAC CC(C/G) GA(A/G) CCG C	(47)
cd3F	916-935	GT(A/C/T/G) AA(C/T) GT(A/C/T/G) AA(A/G) GA(A/G) AC(A/C/T/G) GG	(78)
cd3aF	916-935	GT(C/G) AAC GT(C/G) AAG GA(A/G) AC(C/G) GG	(114)
nirS4F	1317-1336	TTC (A/G)TC AAG AC(C/G) CA(C/T) CCG AA	(12)
nirs4R	1317-1336	TTC GG(G/A) TG(C/G) GTC TTG A(T/C)G AA	(12)
R3cd	1322-1341	GA(C/G) TTC GG(A/G) TG(C/G) GTC TTG A	(114)
R4cd	1636-1654	CGT TGA ACT T(G/A)C CGG T(C/G)G G	(47)
nirs6R	1638-1653	CGT TGA ACT T(A/G)C CGG T	(12)
***nir*K**			
Cunir3	504-521	CGT CTA (C/T)CA (C/T)TG CGC (A/C/G)CC	(22)
nirK1F	526-542	GG(A/C) ATG GT(G/T) CC(C/G) TGG CA	(12)
F1aCu	568-584	ATC ATG GT(C/G) CTG CCG CG	(47)
R3Cu	1021-1040	GCC TCG ATC AG(A/G) TTG TGG TT	(47)
nirK5R	1023-1040	GCC TCG ATC AG(A/G) TT(A/G) TGG	(12)
***nos*Z**			
Nos661F	303-320	CGG CTG GGG GCT GAC CAA	(103)
nosZ-F	1169-1188	CG(C/T) TGT TC(A/C) TCG ACA GCC AG	(65)
Nos1773R	1396-1415	AAC GA(A/C/G) CAG (T/C)TG ATC GA(T/C) AT	(103)
nosZ1622R	1603-1622	CG(C/G) ACC TT(C/G) TTG CC(C/G) T(C/T)G CG	(114)
nosZ-R	1849-1869	CAT GTG CAG (A/C/G/T)GC (A/G)TG GCA GAA	(65)

denitrifying communities in soil and rhizospheres, based on these types of markers, have been reviewed and discussed in Philippot and Hallin , 2006 (90).

The effects of seasonal variations and plants on *nir*K possessing denitrifying communities have been demonstrated in soils (16). In the context of treatment wetlands, very little is known on the community composition of denitrifiers, as well as their degree of spatial or time variation, and

only few studies exist exploring denitrifying communities in wetlands. In one of them, *nos*Z and *nir*S gene surveys have been used to evaluate the effects of plant distribution and hydrological factors on the spatial distribution of denitrifying communities in sediments along a CW system in Sweden (63). Impact of plant species on the community composition of denitrifying bacteria has also been recently investigated with a special interest in the effects of an invasive cattail species (*Thypha* x *glauca*) in a coastal freshwater wetland (3).

1.3.4. Methods for surveying Ammonia Oxidizing Bacteria

The first step of nitrification is catalyzed by ammonia oxidizing bacteria (AOB), a group of gram negative chemolithotrophic bacteria, which obtain energy and reducing power from ammonium, and generally fix atmospheric CO_2 through the Calvin cycle (9, 92).

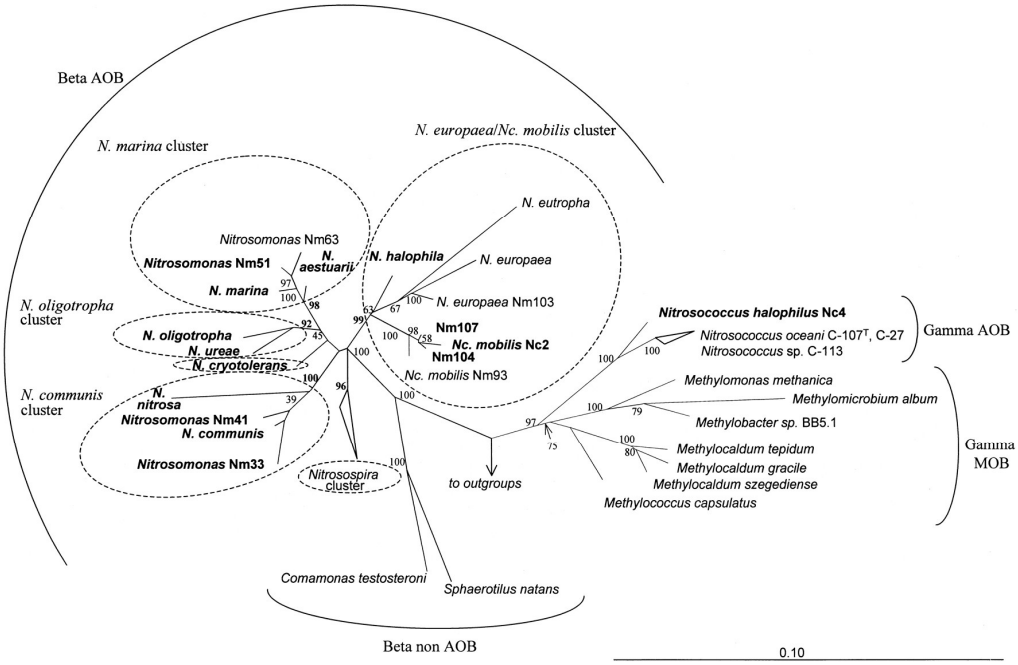

Figure 7. Phylogenetic 16S rRNA tree reflecting the relationships of AOB and several non-AOB reference organisms. MOB, methane-oxidizing bacteria. From Purkhold *et al.*, 2000 (94).

They are classified into two monophyletic groups, the gamma (*Nitrosococcus* sp. and relatives) and beta proteobacteria (*Nitrosomonas* sp. and *Nitrosospira* sp.) groups (Figure 7). The genera *Nitrosolobus* and *Nitrosovibrio* are included within the *Nitrosospira* group. The fact that all the AOB

described belong to beta and gamma *Proteobacteria* subdivisions has allowed them to be studied with molecular markers related to the 16S rRNA gene, although the high sequence homology hampers the analysis at the species or strain level.

Other systems targeting functional genes such as the subunits of the ammonia monooxygenase (AMO) enzyme (Figure 8), responsible for converting ammonia to hydroxylamine, have been developed. Although *amoA* based trees are generally congruent, topologies are not identical and 16S rRNA has been demonstrated to possess higher resolution in comparison to *amoA* analysis (68, 93). The analysis of AMO encoding genes has been extended to *amoB* and *amoC* subunits (21, 85, 94)(Table 5).

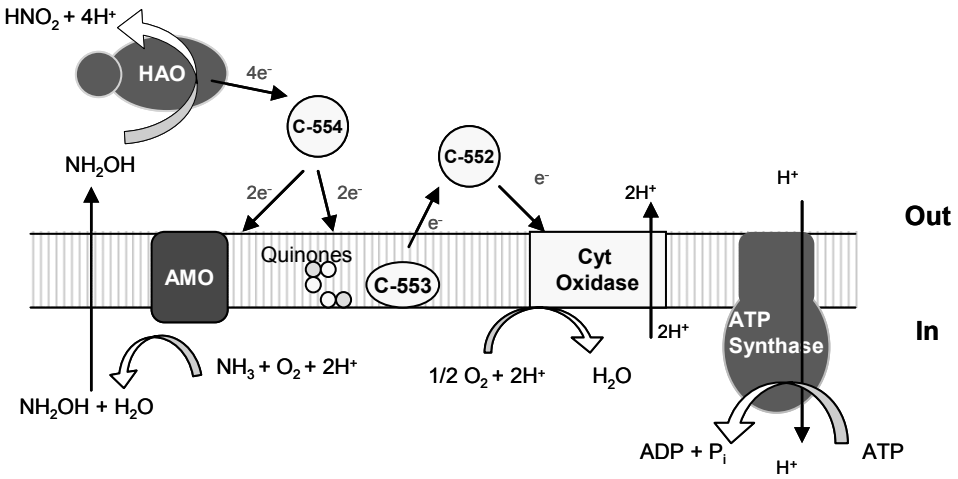

Figure 8. The first step in the nitrification process. Ammonia is oxidized in two sequential steps by the enzymes ammonia monooxygenase (AMO) and hydroxylamine oxide-reductase (HAO). Adapted from Timothy Paustian, University of Wisconsin-Madison, 2000 (www.bact.wisc.edu/microtextbook)

The above described genes allowed the analysis of the distribution and dynamics of AOB communities connected to variations in effluent irrigation in soil, divergent properties of landfill leachates, and the presence of plants. (17, 24, 87, 89, 112). The diversity of AOB communities has not been frequently investigated in FWS-CW. In a vertical flow constructed wetland, *amo*A gene DGGE fingerprints revealed no significant spatial variations of AOB communities (117). In another constructed wetland, based on the DGGE of the 16S rDNA gene, the AOB communities did not show changes in diversity or stability neither as a result of different substratum addition, nor near

the root area of *Phragmites australis*, although certain substrata had an influence on their activity (45).

Table 5. PCR primers used in environmental studies to specifically target bacterial and archaeal groups of ammonia oxidizers.

Targeted gene	Primer	Primer sequence (5'-3')	Ref.
• 16S rDNA	CTO189AB-F CTO189C-F	GGA GRA AAG CAG GGG ATC G GGA GGA AAG TAG GGG ATC G	(67)
	CTO654R	CTA GCY TTG TAG TTT CAA ACG C	
• Ammonia monooxygenase, subunit A	amoA-1F	GGG GTT TCT ACT GGT GGT	(101)
	amoA-2R	CCC CTC KGS AAA GCC TTC TTC	
• Ammonia monooxygenase, subunit B	amoB-Mf	TGG TAY GAC ATK AWA TGG	(21)
	amoB-Mr	RCG SGG CAR GAA CAT SGG	
• Archaeal ammonia monooxygenase, subunit A	Arch-amoAF	STAATGGTCTGGCTTAGACG	(43)
	Arch-amoAR	GCGGCCATCCATCTGTATGT	

1.4. Methods for assessing nitrification and denitrification activity

Diversity studies need to be complemented with activity data, to better understand the relationship between the structure and function of microbial communities. Determining metabolic rates under controlled laboratory conditions provides an estimate of the inherent activity of a given sample, if performed under the ideal conditions for the activity. Although in most cases laboratory experiments do not directly reflect the real activity in the field, they provide necessary data to evaluate the individual effects of different environmental parameters or to compare potential activities in completely opposite situations.

Facing *actual* versus *potential* rates for a given environment is therefore important since their correlation may be indicative of management failures in constructed systems. Moreover, experimental activity analysis allow for a precise comparison of samples without the side-effects of

uncontrolled environmental variables and so may be useful to determine which sites, and under which biochemical conditions, maximum treatment efficiencies may be achieved in a CW.

Extensive research on both nitrification and denitrification activities has been conducted in treatment wetlands, along spatial as well as temporal series. Research has also been focused on different surfaces available to support microbial activity, i.e. the sediment layer, the water column, the periphyton or the rhizosphere, in order to address the influence of hydrological, chemical and biological factors of the nitrogen removal potential (41, 42, 60, 61, 98, 104).

From a methodological point of view, *in situ* experiments are far more complex and often entail the use of closed chambers or specially designed mesocosms. Both *in situ* and laboratory experiments can be monitored in several ways to obtain kinetic data and information. Some of the most commonly used methods are the following.

Calculation of Mass Balances

One simple approach to estimate nitrification and denitrification activities is based on the measure and evolution of NO_3^-, NO_2^- and NH_4^+ concentrations in the liquid phase. This can be easily done in laboratory *microcosms* carried out under ideal conditions for the process of interest in which additional or side metabolic processes are minimized.

In nitrification assays, requirements for optimal activity imply adding NH_4^+ at saturating concentrations and full aerobic conditions. The production of NO_2^- and NO_3^- in incubations is considered to occur due to nitrification activity and the NH_4^+ oxidation and NO_2^- oxidation rates can be easily calculated by linear regression kinetics providing no significant increase in biomass occurs within the incubation period.

The loss of oxidized nitrogen forms (NO_2^- and NO_3^-) can be assumed to occur due to denitrification activity, although other processes such as DNRA or assimilative uptake may contribute to NO_3^- consumption in the incubations and must be kept to a minimum. Denitrification measures usually imply the addition of organic matter, nitrate as an alternative electron acceptor and anaerobic conditions.

Direct denitrification measurements

Alternatively, the acetylene inhibition method can be used to measure denitrification activity. Developed independently by Balderston (8) and Yoshinari (130), this approach measures the nitrogen loss as N_2O accumulation. The nitrous oxide reductase activity is blocked under an

atmosphere with 0.1 to 10% acetylene. This technique has been satisfactorily applied in *in situ* activity analysis and in field controlled measurements using closed chambers but also in intact sediment cores and water-sediment slurries. The method has also been widely used for *ex situ* applications in which activity measures from the pool of enzymes present in a sample at the collection time are provided (109, 116). The production of N_2O in gas samples is determined in a gas chromatograph equipped with an electron capture detector.

The method has limitations associated mainly with C_2H_2 diffusion in soils and sediments, degradation by bacteria, and inhibitory effects on other processes such as nitrification, which together can interfere in the lectures and lead to erroneous estimates of the potential rates (62, 100).

Isotope N-labelled methods

Other methods are available for estimating the nitrification and denitrification rates, such as using stable N isotopes, both in laboratory incubations and field measurements. Using these methods involves adding ^{15}N labelled compounds in the form of NO_3^-, NH_4^+ or more complex fertilizers, and the subsequent quantification by mass spectrometry of the ^{15}N-labelled generated gas compounds. These methods are mostly limited by the high cost of the isotopes and possible impacts of N addition on the environment. Radioactive isotope ^{13}N-labeling methods have also been described but are restricted to laboratory measures for environment safety reasons.

Nitrification activity can also be estimated using the isotope-dilution technique (66). ^{15}N-NO_3^- is added to samples, and nitrifying bacteria dilute the pool of ^{15}N-NO_3^- by oxidizing ^{14}N-NH_4^+ to ^{14}N-NO_3^-. This dilution is considered to be proportional to nitrification. ^{14}N-NO_3^- and ^{15}N-NO_3^- are measured after being converted to N_2 by using a denitrifying culture and analyzing $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ produced with mass spectrometry (33).

It is worth mentioning that the extrapolation or inference of actual, *in situ*, metabolic activity from potential activity measurements can suppose a controversial aspect since many factors (i.e. redox status, chemical compounds, network of environmental variables) that cannot be totally controlled may intervene in determining the former.

1.5. The Empuriabrava CWS

The Empuriabrava CW is located in a coastal region in the NE of Spain, near a tourist urban settlement and immediately adjacent to the Natural Reserve of the *Parc Natural dels Aiguamolls de l'Empordà* (PNAE). The Empuriabrava CW is part of a project to enhance water treatment and preserve the ecological balance of the natural reserve.

The system was set in operation in May 1998, and functions as a reception system for the secondary effluent coming from an adjacent WWTP. Its aim is to perform a final polishing of treated water, and more specifically to remove nitrogen to promote water reuse.

Figure 9. The Empuriabrava Wastewater Treatment Plant and adjacent FWS constructed wetland. Photograph: Ll. Sala.

The treatment plant mainly consists of two carrousel type aerobic-anoxic piston flow reactors followed by secondary settlers and an aerobic maturation pond. The constructed wetland is made up of three vegetated cells of 160 m x 50 m, with an average depth of 0.5 m in which the water flow is evenly distributed and runs towards an outlet channel. Water from the three cells is transported to a common larger, shallower area, the Europa Lagoon (4.5 ha, 0.2 m average depth). The water in this lagoon flows from the centre to the shores, where it is collected by a weir and sent to the El Cortalet lagoon, located within the PNAE. Other uses of treated water include the flooding and irrigation of a pitch and putt recreational complex.

The macrophytic communities found in the wetlands are mainly reeds (*Phragmites australis*) and cattails (*Typha* sp.), introduced with rhizomes from nearby natural wetlands, which form perfectly separated communities in the centre and along the edges of the wetland cells.

Though WWTP effluent quality parameters comply with limits stipulated by legislation, the treatment performance in the CW does not reach optimal removal rates unless a completely nitrified effluent is obtained, so the entrance of water in the wetland when ammonia concentrations exceed 7 ppm is prevented by an automatic by-pass, in which case it is redirected to its original discharge point located in the Muga River. This readjustment of the operational criteria in situations of excessive nutrient input help maintain the wetlands in a healthier state and allow algae, hydrophytes and protozoa to develop better. Therefore, the water quality parameters can be improved even more by clarifying the effluent, reducing faecal coliforms and preventing avian botulism outbreaks.

The wetlands are subjected to strong seasonal variations in both the flow and the respective content of nitrate and ammonia of the incoming water due to intense tourism during the summer months. Except for occasional irregularities, the wetlands perform satisfactorily and consistently over the year, efficiently removing nitrogen which permits environmental reuse of 70-80% of the secondary effluent.

Since it was set up in 1998, the system has become perfectly integrated into the landscape, offering the possibility of using the wetlands for recreational activities, such as bird-watching. The CWs create a habitat for many waterfowl species, therefore contributing to increased biodiversity and the ecological value of the area. Furthermore, other benefits derived from the CWs have spread to the entire surrounding area, such as the substantial improvement in the nearby beach, which

became swimmable after the construction of the WWTP in 1995 and in 1998 reached the highest bacteriological quality standards, which have been upheld until the present.

Regardless of some minor actions, such as macrophytes control in the Europa Lagoon to shape a particular habitat for waterfowl and maintaining the visibility of the hides placed for bird-watching, the Empuriabrava CW involves minimum, if any, maintenance work (102).

One remaining challenge is to upgrade its performance in order to sustain the treatment efficiency and achieve satisfactory nitrogen removal rates throughout the year, even during critical summer months and occasional events of high nutrient input.

OBJECTIVES

Our main goal was to gain insight in the effects of environmental conditions and the presence of emergent vegetation on the nitrifying and denitrifying microbial communities in a free water surface constructed wetland (FWS-CW). The wetland studied as a case model is located in Empuriabrava (Girona, NE Spain) and receives the effluent water of a wastewater treatment plant. The understanding of the critical factors controlling the nitrogen removal will at last permit to develop better management guidelines to enhance performance and efficiently tackle with nutrient peaks occurring during the most critical period in summer.

Specific objectives:

1) To analyze the influence of plant species and operational variables in the diversity of nitrifying and denitrifying bacteria in the sediment surface.
2) To estimate the potential nitrification and denitrification activities of sediments and rhizospheres of main plant species, *Typha sp*. and *Phragmites australis*.

3 RESULTS

The spatial distribution and function of denitrifiers and ammonia oxidizers have been studied focusing on singular aspects of the treatment wetland studied. These aspects are thought to intervene in the dynamics of the underlying microbial communities, and control the nitrogen removal processes. The following section compiles the studies performed with this purpose.

The wetlands, as engineered systems, are subjected to a multitude of external variations at an accelerated rate. These include the effects of natural environmental disturbances, and also man-controlled switches in the operation. Apart from these externally controlled parameters, a wetlands' inherent heterogeneity is presumed to exist between the distinct compartments found, that is, the water, the sediment and the vegetation. Each compartment constitutes a singular microhabitat harbouring characteristic physicochemical and physiological conditions that influence the microbial assemblages capable of thriving or prospering in a certain area. The aim of our work was to elucidate how this complex network of factors governs the development and function of the microbial groups taking part in the nitrogen removal, and provide an integrated approach to assess the removal opportunities within the wetlands.

In the first part, the diversity of denitrifying communities was explored in the sediments of the CW, focusing on the effects of the seasonal hydrodynamics. It is worth mentioning that these studies were performed using samples collected from the inception of the treatment wetland, informing about situations that can be repeated in new installations to be built. The switches in denitrifying communities caused by the emphatic variations in the flow regime, as well as in the nitrogen load and ammonia to nitrate ratio were evaluated through a large time series by a molecular approach based on the analysis of *nir*S gene clone libraries. Clone sequencing revealed a wide diversity of denitrifiers in the wetlands, and the existence of numerous novel sequences distantly related to cultivated bacteria. Moreover, higher diversity indices correlated to situations of high nitrogen load.

Then, a summary of the CW performance over years 2004 to 2006 is given to provide the necessary background. Details regarding the operation of the wetland, the degree of efficiency achieved in the removal of nitrogen, and occurrence of incidents in its performance throughout this period are reported in order to contextualize the next studies.

The following part shows both molecular (T-RFLP analysis of *nir*S genes) data with a special emphasis on the effect of plant species. The spatial heterogeneity within the wetlands was studied in the different compartments that are susceptible to microbial colonization, the bulk sediment and the rhizosphere in vegetated areas. In addition, our goal was to determine whether plant modulated biochemical properties in the root surroundings affected the community composition of denitrifiers. Communities were characterized by fingerprinting patterns and changes in the molecular structure were analyzed comparing sediments and rhizospheres of *Phragmites australis* and *Typha* sp. Furthermore, whether plants contributed to denitrification metabolic capacity was assessed by *in vitro* potential activity measures. Plants undoubtedly appeared to promote denitrification when results are expressed in terms of potential activities per unit dry weight. Although denitrifying communities in sediments or rhizospheres could be clearly discriminated, they were shown to vary from year to year. Large scale environmental variables were therefore considered decisive in shaping community structure, whereas plants were crucially involved in stimulating the function of this functional group.

Finally, in the last part, we went further into determining plant specific shifts in the microbial communities, including the analysis of ammonia oxidizing bacteria. The occurrence of minority

yet characteristic denitrifying strains in plant patterns when *nir*S was used as a selective marker led us to map denitrifying communities with other markers such as the *nos*Z gene, encoding the last enzyme in the denitrification cascade. While the AOB communities were found to be highly homogenous in either sediments or rhizospheres, the nitrification activity was obviously stimulated in the vicinity of roots. Plant specific effects on denitrifiers were finally reassessed by activity and molecular analysis, taking into account the possible effect of hydraulic and seasonal fluctuations. With this objective we monitored the switches in community composition along a temporal scale associated to drying events in the wetland. These situations were the result of the application of high and low water retention times and nutrient inputs.

3.1. DIVERSITY OF THE NITRITE REDUCTASE GENE NIRs IN THE SEDIMENT OF A FREE-WATER SURFACE CONSTRUCTED WETLAND

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Diversity of the nitrite reductase gene *nirS* in the sediment of a free-water surface constructed wetland

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Summary. The diversity of the nitrite reductase gene *nirS* was studied in the bulk sediment of a free-water surface constructed wetland (FWS-CW) located next to the Empuriabrava wastewater treatment plant (WWTP), in Castelló d'Empúries (Girona, NE Spain). The study period extended from the inception of the treatment wetland, in June 1998, until March 1999 and comprised periods of relatively high nitrate and ammonium concentrations at the influent and low nitrate-removal efficiencies. To evaluate *nirS* diversity, partial gene sequences were obtained by cloning of the respective PCR products. Rarefaction curves based on DOTUR analyses of the deduced amino-acid sequences predicted a greater diversity of *nirS* genes in samples containing higher ammonium concentrations. Estimated Shannon-Weaver indices of the four cloned samples showed a positive relationship with the $N\text{-}NH_4^+/N\text{-}NO_3^-$ ratios measured at the FWS-CW inlet. Identities between the deduced amino-acid sequences and those previously deposited in public databases ranged from 72 to 97%. Phylogenetic analysis based on these deduced sequences grouped 165 *nirS* clones in seven main clusters according to high similarity indices. Up to 60% of the clones clustered together in a highly homogeneous group with little homologies to any sequence retrieved from cultured representatives. Moreover, prevailing environmental conditions appeared to select for particular denitrifying populations, e.g., with respect to ammonium load and nitrogen removal efficiencies. This observation is of particular interest for the management of treatment wetlands, in which only slight variations in the theoretical denitrification potential of the system can occur. [**Int Microbiol** 2007; 10(4): 253-260]

Key words: *nirS* gene · denitrification · free water surface constructed wetland · sediments

Introduction

Constructed wetlands are engineered ecosystems designed for the unconventional on-site treatment of diffuse or non-point polluted water, such as often derived from industrial and rural management or as wastewater from small settlements. The two main types of constructed wetlands are free-water surface and subsurface flow systems [18]. Free-water surface constructed wetlands (FWS-CWs) can be used to treat the wastewater of large municipalities, especially those located in temperate climates. Treatment processes within a FWS-CW mainly involve the use of vegetation, which promotes the massive growth of bacteria through the production of organic-matter exudates and by oxygen diffusion to the sediments surrounding the root system [24]. In addition, emergent plants slow down the rate of water flow and thus are able to trap many pollutants, which may be further transformed and removed from the water by microbial communities [5,7,16].

Organic-matter reduction and nitrification/denitrification processes are among the major purposes of FWS-CW. Denitrification, in which there is a net loss of nitrogen from water, takes place ubiquitously in soils and sediments. It consists of the stepwise biological reduction of nitrogen oxides to

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molecular nitrogen, coupled to the phosphorylation of elements in the electron-transport chain. Denitrification has become an issue of global concern, since some of the produced intermediates, i.e., nitric and nitrous oxides, may contribute to the degradation of the ozone layer and to act as greenhouse gases [14].

Denitrifying organisms are present in a broad range of taxa, including *Bacteria* and *Archaea*; in addition, denitrification is carried out in the mitochondria of several species of fungi [30]. True denitrifiers differ from nitrate-reducing organisms in that the former can reduce NO_2^- to NO and finally to nitrogen gas, thus removing nitrogen from water. Nitrite reductases are responsible for this step and can be found among denitrifiers in two structurally different but physiologically and functionally equivalent enzymes: Cu-containing nitrite reductases and heme-containing cd_1 nitrite reductases, encoded by the *nirK* and *nirS* genes, respectively [9,30]. The screening of functional genes involved in denitrification as molecular markers of this process has proven useful to study the diversity of denitrifying bacteria in different environments. Specific PCR primers for *nirK*, *nirS,* and *nosZ* (the latter encoding nitrous oxide reductase) have been used to survey the composition of the denitrifying community in samples from aquatic and soil habitats and to establish correlations with environmental variables [3,4,6,12,13,25]. Moreover, the information gained from the cloning and sequencing of *nirS* and *nirK* genes has provided a comprehensive measure of the community diversity of environmental samples and has revealed the existence of novel denitrifying bacteria that have diverged completely from previously cultured bacteria [3,8,11].

Constructed wetlands are systems designed to improve water quality and yield parallel benefits such as increasing the availability of food and habitats for wildlife as well as for aesthetic recreation. Therefore, intensive research on the denitrifier communities in FWS-CWs and the changes in community composition that occur due to natural or human impacts on wastewater treatment systems is essential to better understand the role of denitrifiers in nitrogen cycling. The aim of this study was to assess the structure of the denitrifying community within a model FWS-CW and to monitor seasonal and human-derived changes in the community through the analysis of *nirS* genes as functional molecular markers.

Materials and methods

Site description and sampling. The FWS-CW of Empuriabrava was set up in the summer of 1998 as a tertiary treatment of a wastewater treatment plant (WWTP). It is located in the vicinity of a highly developed tourist resort in Castelló d'Empúries (Girona, NE Spain) and was originally designed to provide water of improved quality for flooding the nearby marsh area of the natural reserve Els Aiguamolls de l'Empordà. The system covers a surface area of 64,802 m^2 with an average depth of 0.5 m and it comprises three parallel cells plus a receiving lagoon. The treatment cells are sparsely covered with vegetation, mainly consisting of reeds (*Phragmites australis*) and cattails (*Typha latifolia*) grouped in independent communities. The wetland receives an annual mean flow of 1600 m^3/day, with strong seasonal variations ranging from 4984 m^3/day (August) to 650 m^3/day (February).

Physical and chemical monitoring of the FWS-CWs were carried out routinely every 15 days. Water samples for chemical analyses (100 ml) were collected directly from the effluent of the WWTP and at the pumping station in the receiving lagoon (see Fig. 1 for locations of sampling points). Conventional standard methods employed in wastewater analysis were used to

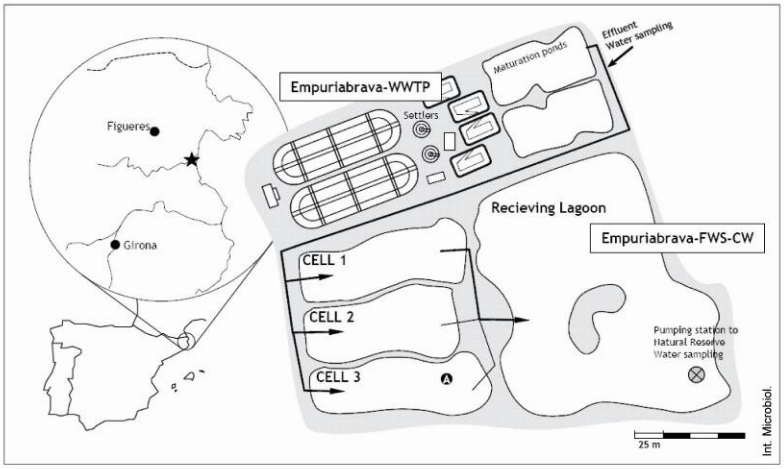

Fig. 1. Schematic map of the Empuriabrava wastewater treatment plant (WWTP) and the adjacent free water surface constructed wetlands system (FWS-CW). Sediment samples were taken at station A. Points for water sampling are marked as Effluent and Pumping station. Solid line shows the preferential water flow.

measure pH and N-NO_3^-, N-NO_2^- and N-NH_4^+ concentrations [1]. Total inorganic nitrogen (TIN) was calculated from the sum of the amounts of nitrate, nitrite, and ammonia.

The sampling station was located 30 m away from the outlet of the third cell of the constructed wetland (Fig. 1). Samples were obtained regularly from the inception of the FWS-CW. All data refer uniquely to four sampling dates, June 1998, October 1998, December 1998, and March 1999. Sediment samples were collected as single undisturbed sediment cores by using a manual sampler mounted with a 9-cm diameter Plexiglas tube, and were transported to the laboratory in a portable ice box in the dark. Smaller sediment cores (2.5 cm diameter) were obtained aseptically and the upper 3 cm were completely homogenized by means of manual stirring. Triplicate 1-g aliquots were placed in sterile 2-ml Eppendorf tubes and stored at –20ºC until processed.

Nucleic acid extraction. Nucleic acids were extracted according to a previously described phenol-chloroform method for the isolation of DNA from wetland sediment samples [20], with the following modifications: 200–400 mg of sediment was added to 2-ml screw-capped vials containing 1.5 g of a mixture of 0.1-mm (diameter) glass and 0.5-mm (diameter) silica beads (BioSpec Products, Bartlesville, OK). The beads were mill-homogenized at maximum intensity (2800 rpm) for two consecutive periods of 45 s in a MiniBeadBeater-8 mill (BioSpec Products). Both chemical and enzymatic treatments and DNA purification and concentration were done following the methods described by Miller et al. for freshwater sediments [20].

PCR amplification of *nirS* genes. The *nirS* gene was amplified in a Geneamp PCR system 9700 (Applied Biosystems, Foster City, CA) with the primer pair nirS1F:nirS6R [2]. PCR reaction mixtures contained 1 × PCR reaction buffer, 2 mM $MgCl_2$, 200 µM total dNTPs, 0.8 mg bovine serum albumin (New England Biolabs, Beverly, MA)/ml, 1 µM of each primer, 10–100 ng of the DNA extracts, and 0.025 U *Taq* polymerase (AmpliTaq DNA Polymerase, Applied Biosystems) in a total volume of 50 µl. After an initial denaturation step of 5 min at 94ºC, amplification reactions were carried out with 10 touchdown cycles of denaturation (30 s at 94ºC), primer annealing (1 min, 60ºC, decreasing 0.5ºC at each cycle), and primer extension (1 min, 72ºC), followed by 30 cycles at an annealing temperature of 57ºC and a final extension step of 10 min at 72ºC. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized after staining with ethidium bromide (0.5 mg/l).

Clone libraries of *nirS*. Four sediment samples were selected and used for an extensive analysis of *nirS* gene diversity as determined through cloning experiments. The four samples were obtained from station B at four different phases of the flooding and colonization of the FWS-CW: June, October, and December 1998, and March 1999. The *nirS* gene was cloned using the pGEM-T Easy vector (Promega, Madison, WI) following the instructions provided by the manufacturer. Ligated products were transformed into *Escherichia coli* DH5 competent cells, and positive transformants were color-screened on LB plates supplemented with ampicillin (100 µg/ml), X-Gal (80 µg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG; 20 mM). Clones were selected for positive *nirS* fragments by PCR using the primer set nirS1F:nirS6R in 20-ml reactions as described above but without the addition of BSA and using a 35-cycle PCR program at a constant annealing temperature of 57ºC. Positive clones were sequenced using vector-specific primers M13F and M13R (Macrogen, Seoul, South Korea). Sequences were manually refined using the BioEdit package [10] and then aligned using ClustalW [28].

Construction of rarefaction curves and phylogeny. DNA alignments of partial *nirS* gene sequences retrieved from clones were used to construct a DNAdist (DOTUR package) DNA distance matrix. Rarefaction matrices and Shannon-diversity and evenness indices were also calculated with the DOTUR package [26]. Cut-off values of 20% sequence divergence were selected, following the recommendations for protein-coding genes. Phylogenetic and molecular evolutionary analyses were carried out with deduced amino-acid sequences and by using the MEGA version 3.0 software. Phylogeny was reconstructed according to the Amino Poisson correction method and by pair-wise deletion using a bootstrap value of 1000. *Pseudomonas aeruginosa* gene sequences *nir*N (D84475) and *nir*F (D50473) were used for comparison.

Nucleotide sequence accession numbers. The partial *nirS* sequences were deposited in GenBank under accession numbers EF558372 through EF558536.

Results

Chemical characterization of the Empuriabrava-FWS-CW. The amount of water flowing through the treatment wetland strongly depended on the sizes of the summer tourist populations in the nearby tourist resorts of Empuriabrava and Roses. Calculated mean residence times varied from approximately 6.3 days in August to almost 33 days in February. The differences in water mean residence time were mainly due to changes in the amount of water flowing through the Empuriabrava WWTP, since there was little influence due to evaporation or changes in the water level at the treatment wetlands. Significant positive and negative correlations ($P \leq 0.05$) were found between wastewater flow and ammonium or nitrate concentration at the influent, respectively (Table 1). Generally, high ammonium concentrations occurred during summer, with maximum point values of 26.5 mg N-NH_4^+/l recorded during July 1998. Nitrate concentration at the influent showed the opposite behavior, with maximum values recorded at low flow rates and during high residence times in the winter and early spring. Nitrite showed little variation throughout the study period. Concentrations of less than 0.3 mg N-NO_2^-/l were invariably recorded at the inlet and outlet sampling stations of the FWS-CW (results not shown). The removal of TIN, calculated in the FWS-CW according to mass-balance equations, varied from 118 to 2737 kg N/month, which corresponded to efficiencies of 25.9 and 91.7% of the nitrogen load. Greater removal efficiencies were measured during the summer.

In contrast to the wide variations in nitrogen concentration, the pH of the water in the Empuriabrava-WWTP remained almost constant, with values around 7.4 (Table 1). However, a measurable increase in pH over the entire study period was measured at the constructed wetland. At the sampling point located at the pumping station, pH records reached mean values of 10.0 during March 1999. The higher pH values recorded at the outlet may have been indicative of high-level metabolic activity.

Table 1. Mean monthly values and deviation of the mean of relevant environmental variables at the Empuriabrava free water surface constructed wetland (FWS-CW) during the study period

	Residence time (days)	pH inlet	pH outlet	NH_4^+ inlet (mg N/l)	NO_3^- inlet (mg N/l)	TIN removal (%)[b]
Year 1998						
June[a]	17.4	7.4 ± 0.2	9.1 ± 0.3	7.2 ± 3.8	6.2 ± 0.6	56.7
July	9.8	7.6 ± 0.1	8.5 ± 0.5	16.1 ± 6.9	2.3 ± 2.4	78.0
August	6.3	7.3 ± 0.1	8.2 ± 0.4	17.2 ± 6.0	1.8 ± 1.1	91.7
September	10.4	7.5 ± 0.2	8.8 ± 0.2	11.2 ± 3.8	3.1 ± 2.4	50.5
October	16.0	7.5 ± 0.3	8.8 ± 0.0	10.5 ± 4.5	3.3 ± 4.0	83.7
November	28.2	7.2 ± 0.1	8.4 ± 0.7	4.8 ± 0.9	7.6 ± 2.0	49.0
December	21.4	7.7 ± 0.2	8.0 ± 0.4	4.6 ± 0.3	9.8 ± 0.2	25.9
Year 1999						
January	19.8	7.3 ± 0.1	8.6 ± 0.0	7.6 ± 2.6	9.35 ± 0.3	55.2
February	33.5	7.9 ± 0.1	9.1 ± 0.5	8.0 ± 2.8	9.43 ± 0.4	49.0
March	28.6	7.6 ± 0.1	10.0 ± 0.3	2.8 ± 1.0	9.30 ± 0.1	68.7
April	19.7	7.6 ± 0.1	9.1 ± 0.1	5.6 ± 0.6	2.63 ± 1.1	75.2

[a]Data collected beginning June 15. [b]Mean monthly removal efficiencies (%) of total inorganic nitrogen.

Analysis of the nitrite reductase (*nirS*) gene clone library. *nir*S sequences were obtained from four sediment samples taken at different times from the sampling station of the FWS-CW treatment cells: the inception of the FWS-CW, June 1998 (JUN98, CloneD library); October 1998, a period of high ammonium content and highly efficient removal rates (OCT98, CloneA library); and December 1998 (DEC98, CloneC library) and March 1999 (MAR99, CloneB library), two periods of relatively high nitrate concentrations. Positive *nir*S clones from each library were randomly selected and completely sequenced. There were few positive clones from the JUN98 sample, although cloning was carried out twice, slightly changing the PCR conditions. Nonetheless, only 15 positive clones could be obtained and sequenced. The effective coverage with respect to overall diversity and number of species was evaluated from rarefaction curve plots (Fig. 2). Sample OCT98 contained the highest richness, resulting in 18 operational taxonomic units (OTUs) out of 68 clones screened. In contrast, although the rarefaction curves could not be saturated for the sample JUN98 and, consequently, fewer OTUs were detected, the estimated Shannon-Weaver (SW) diversity index was comparable to that determined in October 1998 (Table 2).

Samples DEC98 and MAR99, representing conditions of higher nitrate concentration at the inlet of the FWS-CW and TIN removal efficiencies of 26 and 69%, respectively, had lower diversity indices.

Deduced NirS amino-acid sequences revealed at least 72% identity to previously deposited sequences in public databases. Phylogenetic analyses produced a relatively high number of groups with low similarity to sequences from previously isolated and cultured microorganisms (Fig. 3). This clustering agrees with previous topologies found for the *nirS* gene in marine environments and marsh soils [22]. Group I (130 clones, 79% of the total) consisted of *nirS* sequences with the lowest identities to known sequences, and thus little relatedness to identified bacteria. In general, a BLAST search for these sequences revealed their high similarity to *nirS* clones retrieved from marine environments. Sequences in subgroup I.a had 78–92% identity to marine clones but showed little homology with any cultured representative. Subgroup I.b comprised clones with similarities to previous sequences of 76–83%, *Azoarcus tolulyticus* (AY078272.1) being the closest cultured reference. Six clone sequences were assigned to subgroup I.c, together with *nirS* sequences of *Ralstonia eutropha* (AF114789), and were 79–97% identical to cloned *nirS* sequences retrieved from activated sludge treatment reactors.

Group II (30 clones) comprised several subgroups supported by high bootstrap values. Members of subgroup II.a were 85–90% related to sequences present in activated sludge reactors and showed little similarity with reported sequences derived from cultured representatives. CloneB46,

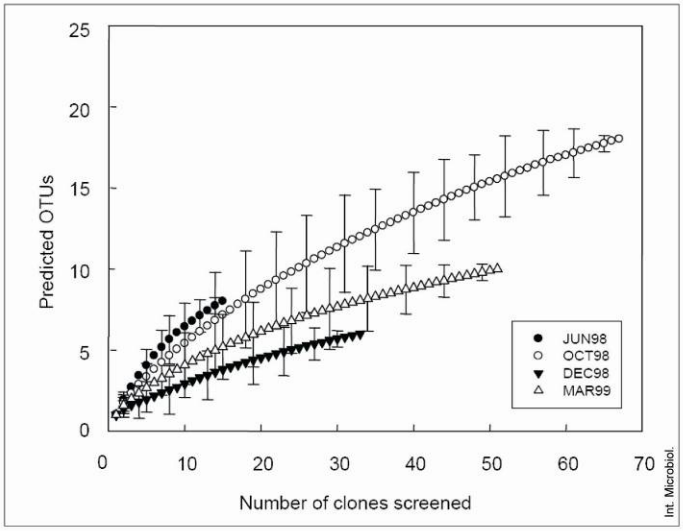

Fig. 2. DOTUR rarefaction curves indicating the diversity of *nirS* gene sequences of individual cloned samples from June 1998, October 1998, December 1998, and March 1999 of the Empuriabrava FWS-CW. Error bars represent the 95% confidence interval.

obtained in March 1999, clustered with *Azospirillum brasilense* and was considered as a single group. Most reference sequences from cultivated microorganisms clustered together with CloneA38, CloneA44, and CloneA68 in group IIb. All environmental sequences derived from the October 1998 sample. Clones included in subgroup II.c shared more than 90% identity with previously known *nirS* gene sequences, mostly retrieved from nitrate- and uranium-contaminated aquifers.

Finally, CloneC8, isolated in December 1998, branched individually in Group III. This clone was found to be closely related to *nirS* sequences retrieved from marine samples, which were previously shown to cluster apart from other *nirS* sequences [3,22].

Table 2. Diversity indices calculated from DOTUR rarefaction analysis using a distance similarity cut-off of 20% of the deduced amino acid sequences of partial *nirS* genes. *S*: number of operational taxonomic units (OTUs) determined based on rarefaction analysis. The number of clones screened is shown in parentheses. *H*, Shannon-Weaver diversity index; *E*, evenness calculated as $E = H/Hmax$, where $Hmax = ln(S)$

	S	*H*	*E*
JUN98	8 (15)	1.933	0.929
OCT98	18 (67)	2.015	0.697
DEC98	6 (33)	0.845	0.471
MAR99	10 (51)	1.360	0.590

Discussion

A cloning-based analysis was used to estimate the composition and diversity of NirS-containing denitrifying bacteria in sediments of the Empuriabrava FWS-CW. The study of denitrifying communities in wastewater treatment systems such as this one is of major importance, since nitrogen removal from water depends mostly on denitrification rates. Although our results are limited to the analysis of *nirS* gene sequences, we also attempted a similar study on *nirK*. Unfortunately, despite the use of different primer sets designed for the analysis of *nirK* genes and reactions carried out at several annealing temperatures, no positive PCR products were obtained from the samples studied. Other reports in the literature have highlighted the difficulties in amplifying either kind of nitrite reductase from certain ecosystems, including upland soils and marine sediments. For example, *nirS* genes could not be detected in a sample obtained from a forested upland soil in Michigan, USA, nor in the rhizosphere of cultivated grain legumes [22,27]. However, *nirK* was detected, albeit not readily, in marine sediments from Puget Sound, Washington, USA, and from the River Colne estuary, in the UK, even though high denitrification rates were assumed to occur in the latter [3,21]. Yan et al. [29] observed different trends in *nirS*- or *nirK*-harboring denitrifier communities retrieved from five contaminated groundwater sites. These authors also estimated inversely related diversity indices for *nirK* and

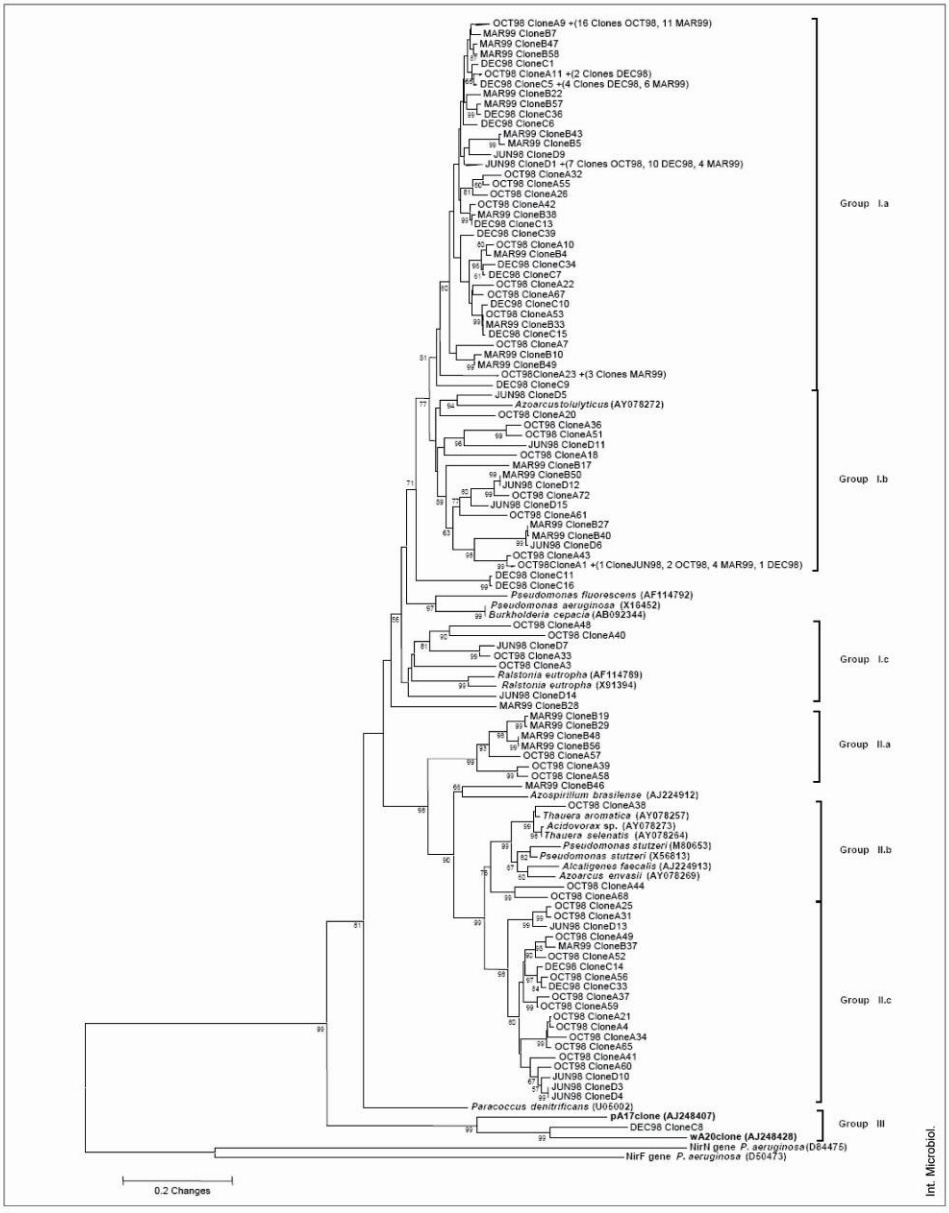

Fig. 3. Neighbor-joining phylogenetic diagram of translated *nirS* partial sequences (ca. 290 residues) from bulk sediment of the Empuriabrava FWS-CW. Amino Poison correction and pairwise deletion were used. Percentage values supporting bootstrap values higher than 50% are indicated at branch points. *nirF* (D50473) and *nirN* (D84475) gene products served as outgroups. Clusters containing >99% identical sequences are indicated by a representative sequence followed by the amount and origins of the other sequences. Selected database sequences and accession numbers are highlighted in bold type.

Table 3. Relative frequencies (%) of partial *nirS* sequences obtained from cloned samples in every group defined from the phylogenetic analysis

	Group I			Group II				
	I.a	I.b	I.c	II.a	II.b	II.c	Group III	Others
JUN98 (n = 14)	14	43	14			29		
OCT98 (n = 67)	51	15	6	4	4	19		
DEC98 (n = 33)	82	3				6	3	6
MAR99 (n = 51)	71	16		8		2		4

nirS genes. This apparent preference of microorganisms harboring either type of nitrite reductase for certain environments might be due to the selection of denitrifying bacteria better adapted to particular environmental conditions. However, the presence of *nirK*-harboring organisms in the Empuriabrava FWS-CW cannot be completely ruled out from the analyses reported here, and other studies, such as selective isolation of denitrifying bacteria, should be conducted in those sediments.

The existence of a widely occurring group of *nirS* sequences that lack any cultured representatives (approximately 80% of retrieved sequences) stresses the need to intensify efforts aimed at the isolation of new species of denitrifiers. The phylogenetic analysis clustered all clones into three main groups. Group I, especially subgroup I.a, contained most of the clones and comprised sequences less closely related to those found in the databases, which suggests that the diversity of *nirS* will increase as new environments and conditions are investigated. The greatest homologies found in this group corresponded to partial *nirS* fragments obtained from either marine sediments or the marine water column [3,17]. This could be due to the scarce studies on denitrifying organism from non-marine environments, which reduces the number of sequences available for comparison. Some of the clones included in Group I, in particular those in subgroup I.c, showed closest homology with *nirS* sequences retrieved from activated sludge treatment reactors and cultured representatives such as *Ralstonia eutropha*. Sequences in Group II represented only 20% of all analyzed sequences, branched together with sequences from well-characterized denitrifying microorganisms, and resembled *nirS* sequences obtained from wastewater treatment reactors [29].

The prevailing environmental conditions, such as the ammonium concentration in the influent entering the constructed wetland or the removal capacity, determine the diversity of the *nirS*-containing denitrifying community. Relative abundances of clones clustering in groups I.a–III varied according to the sampling date (Table 3). Sample JUN98, obtained at the inception and during flooding of the FWS-CW, contained the highest relative number of clones belonging to Groups I.b and II.c, which were poorly represented in the other cloned sequences. This suggests that bacterial diversity is mainly affected by the water of the WWTP itself or the original microbial diversity of the impermeabilization bed covering the bottom of the wetland. The highest diversity indices were detected in the June and October 1998 samples, when the FWS-CW influent was characterized by a high ammonium/nitrate ratio. Although this condition was less favorable for high specific denitrification rates, the removal efficiencies of TIN under other conditions have been reported to fall in the same range. By contrast, in the winter, nitrate supplies and retention times in the wetland were higher. As shown by the diversity of the *nirS* gene, the high nitrate supply and the retention times favor the onset of a few defined populations, such that more than 85% of the retrieved sequences clustered in groups I.a and I.b. Using similar molecular techniques, other authors previously reported variations in the community composition of denitrifying bacteria as a response to environmental factors [15]. Nitrate and oxygen were shown to have a decisive effect on the structure of denitrifier communities in the continental margin sediments of the oxygen-deficient zone of the Pacific Coast of Mexico [19]. Moreover, Castro-González et al. were able to demonstrate variations in the relative abundances of *nirS* terminal restriction fragments along O_2, NO_3^-, NO_2^- and depth gradients in the water column of the oxygen minimum zone in the Eastern South Pacific [6].

The study of shifts in the diversity and composition of denitrifying and other microbial communities [23] is of particular interest in engineered systems, such as the constructed wetland studied here, that face high seasonal variations. In treatment systems implemented mostly for the removal of nitrogen, the complete potential of the denitrification activity must be expressed regardless of changing conditions, including lower nitrification rates in the WWTP and a high ammonium concentration at the inlet of the constructed wetland.

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3.2. THE PHYSICAL AND CHEMICAL SCENARIO: TIME VARIATION OF MAIN PHYSICOCHEMICAL VARIABLES AND PERFORMANCE OF THE CONSTRUCTED WETLANDS DURING 2004-2006

The physical and chemical scenario: Time variation of main physicochemical variables and performance of the Empuriabrava Constructed Wetlands during the period 2004-2006.

Summary

The Empuriabrava Constructed Wetlands were designed as a water polishing system to treat the secondary effluent from the Empuriabrava WWTP. The treatment plant was designed to provide the wetland with a completely nitrified and partially denitrified effluent, with very low concentration of ammonia. In this sense, the wetland system was basically committed to decrease the concentration of nutrients, mainly in the form of nitrate and phosphorus. A free water surface wetland was chosen as the preferable option since a positive impact in the aesthetics of the zone together with providing new flooded areas to increase the quality of wildlife habitat, were among the main objectives desired for the new treatment facility located next to the natural marshes of *Els Aiguamolls de l'Empordà*. The latter objectives have been successfully achieved; the cells of the Empuriabrava FWS-CW receive a constant flow of visitors and constitute one of the defined spots for bird-watching in the Natural reserve. But, as will be shown in the following section, the treatment expectations of the FWS-CW have not been completely accomplished.

The purpose of this section is to give an overview of the performance of the constructed wetlands during the 2004 to 2006 period. The previous section was dedicated to some microbiological aspects during the inception and initial years of operation of the constructed wetland, with a special emphasis on the diversity of denitrifiers in the sediment portion. The use of detailed information on physicochemical data obtained both at the effluent of the WWTP and at different sampling points at the CW will be used to better describe the performance of the system and explain some of the irregularities or remarkable incidents in its operation that occurred during this period. The presented data will provide the desirable framework for the correct understanding of the wetlands' operation and the reasoning for the microbiological study performed.

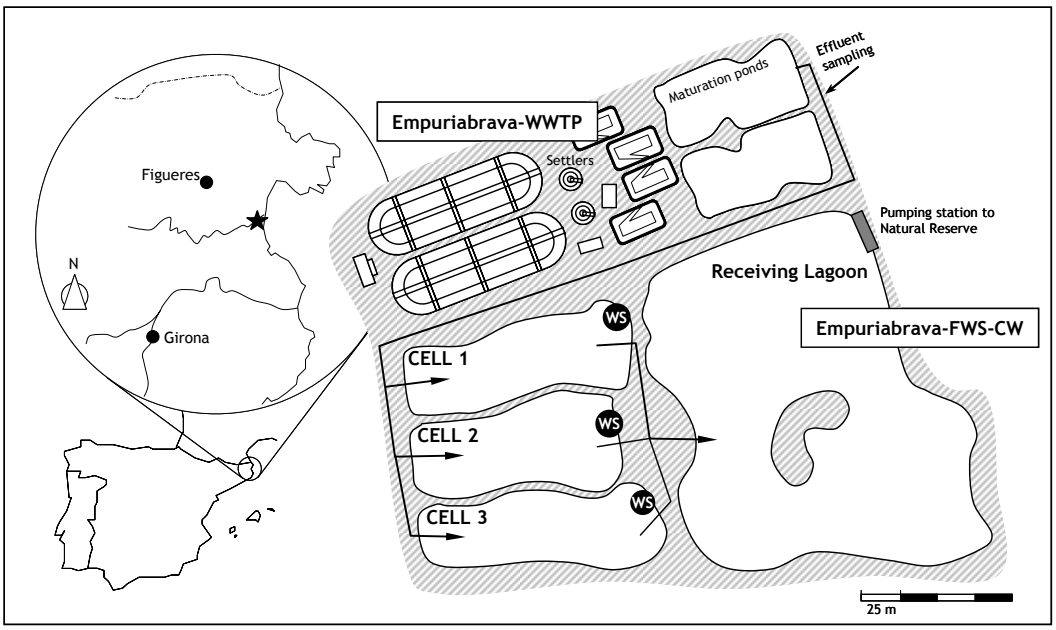

Figure 1. Empuriabrava WWTP and adjacent free flowing surface constructed wetlands system (CWS). Location of water sampling sites within the wetland cells is indicated by WS dots.

Vegetation coverage

Vegetation distribution and density is a key parameter for the management of surface-flow constructed wetlands (14). Vegetation in the treatment cells of the Empuriabrava FWS-CW (Figure 1) consists mainly of common reed (*Phragmites australis*) and cattail (*Typha* sp.). Both species are mainly grouped in independent communities and sparsely planted along the flow direction. The surface coverage of *Phragmites australis* and *Typha* sp. stands was estimated from aerial images obtained from the *Institut Cartogràfic de Catalunya* (ICC, www.icc.es) and digitally analyzed with the ImageJ software v1.38 (NIH, US). Areas of poor image quality were inspected *in situ* to define coverage of different plant species.

Vegetation coverage was 40.9% in of the cell surface in Cell 1, 33.2% in Cell 2 and 38.3% in Cell 3. *Phragmites australis* and *Typha* sp. stands were heterogeneously distributed in the three treatment cells and formed separate communities in most areas (Figure 2). Mixed stands of both plant species were present only in Cells 1 and 2 and accounted for 11.1 and 18.9% of the vegetated area,

respectively. Cell 1 was predominantly covered with cattail, 67% of the total vegetated area, which was mainly distributed downstream the treatment cell. Several attempts to increase the density of vegetation, mainly *Phragmites australis*, at the inlet of Cell 1 have been made over the last years with a poor success. The vegetation in Cells 2 and 3 is predominantly located at the inlet area, sparsely covering zones downstream the central parts of the cells. Cell 2 showed a large coverage of *Typha* sp. (70.4%) and Cell 3 was dominated by areas covered by *Phragmites australis* (62.5% of the total vegetated area). The coverage and distribution of the vegetation offered an interesting set of conditions within a small area in three cells of similar morphology.

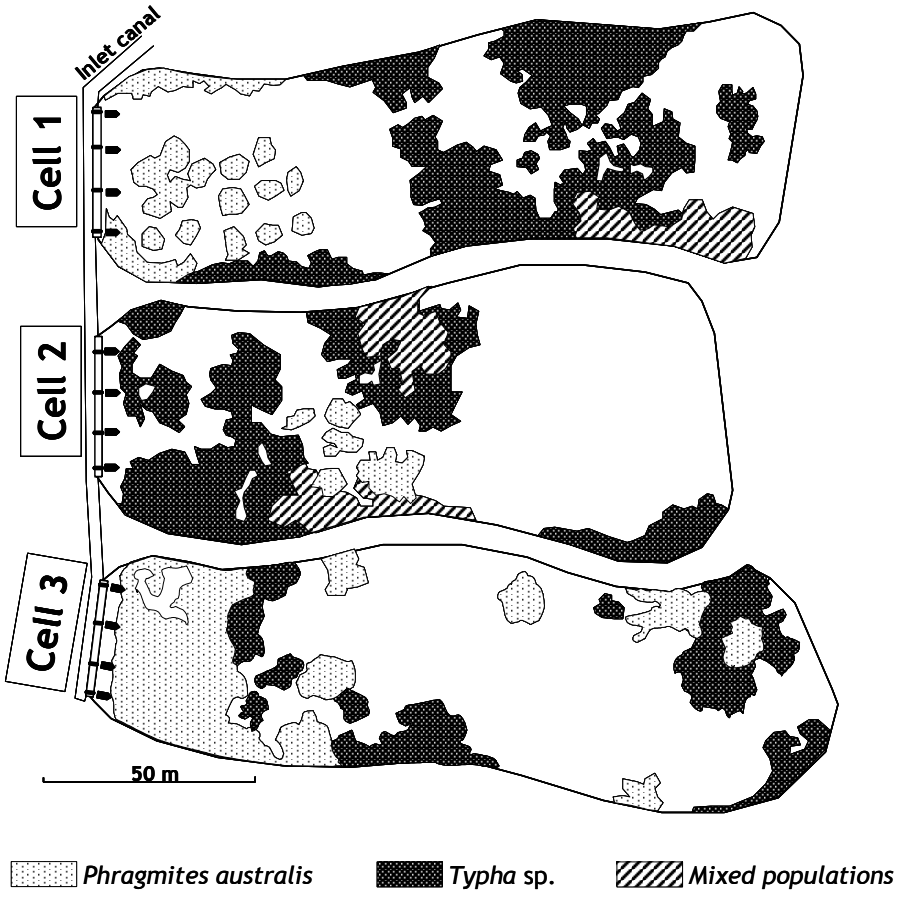

Figure 2. Map of the plant coverage and species composition in the three parallel treatment cells of the Empuriabrava CWs. Areas corresponding to cattail (*Typha* sp.) or reed (*Phragmites australis*) appear in different patterns.

Physical and chemical characterization of the FWS-CW

Physical and chemical monitoring of water at the inlet and outlet points of the treatment cells were done every two weeks at the defined positions (Figure 1). Water samples for chemical analyses (100 ml) were collected directly from the secondary effluent of the WWTP and at an end point of the three treatment cells. pH, conductivity, biological oxygen demand (BOD), oxygen saturation, and concentrations of soluble phosphate (SRP), nitrate, nitrite and ammonium, were determined using conventional standard methods for wastewater analyses (APHA, AWWA and WEF (1995)). Nitrogen and phosphorous removal efficiencies were estimated from mass balance equations considering on-line flow measures and differences of nutrient concentration at upstream and downstream sites of the treatment cells.

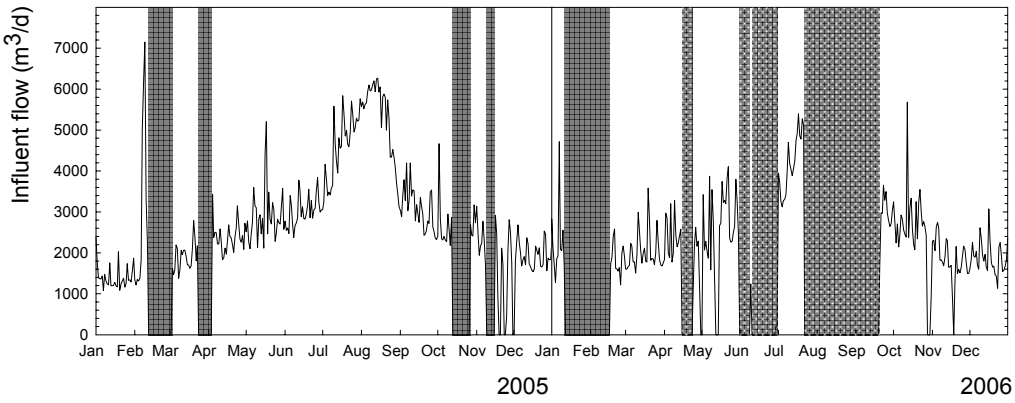

Figure 3. Variations in the water load (m^3/d) to the Empuriabrava CW system during years 2005 and 2006. Periods of flow interruption appear shaded and the recording of ammonia concentrations in the inlet superior to 7 ppm is indicated by a dotted pattern.

Annual mean flows are around 2,500 m^3 day^{-1} (2005), and 1480 m^3 day^{-1} (2006) but exhibit a strong seasonal variability ranging from monthly averages of 1,000 and 799 m^3 day^{-1} (February 2005 and 2006, respectively) to almost 6,000 and 2,848 m^3 day^{-1} (August 2005 and July 2006).

The hydraulic load to the CW showed a marked seasonal variation on the three consecutive years. The variations in flow rates ranged from 963 m^3/d (November) to a maximum value of 6,296 m^3/d during August, in 2005; or from 200 m^3/d (June) to 5,682 m^3/d (July) in 2006 (Figure 3). The

observed seasonal increase in flow is mainly due to the tourist activity in the region, which causes a significant population increase, especially in August, and demands for an effective treatment of a greater wastewater load. Many of the flow interruptions observed during the studied period occurred due to internal operational criteria restrictions or limitations in the WWTP performance. As an example, the poor sedimentation and the presence of mud at the WWTP effluent limited flooding of the CW from January to mid February 2006. However, most of flow interruptions into the CW, for example those recorded during summer 2004 and from the 22nd of July to the 20th of September 2006, obey to unusually high ammonia concentration in the WWTP effluent. Flow interruption to the system at ammonia concentration levels above 7 mg/L $N\text{-}NH_4^+$ is used to prevent the flood of the wetland with low oxygenated water and avoid the development of extreme anoxic conditions in the sediment. Anoxic conditions have derived in localized outbreaks of avian botulism in the area causing bird mortality in the past. Some severe episodes of anoxia, July-August 2006, have forced the complete drainage of the wetland cells to prevent the spreading of botulism to adjacent natural marshes and protect the indigenous fauna.

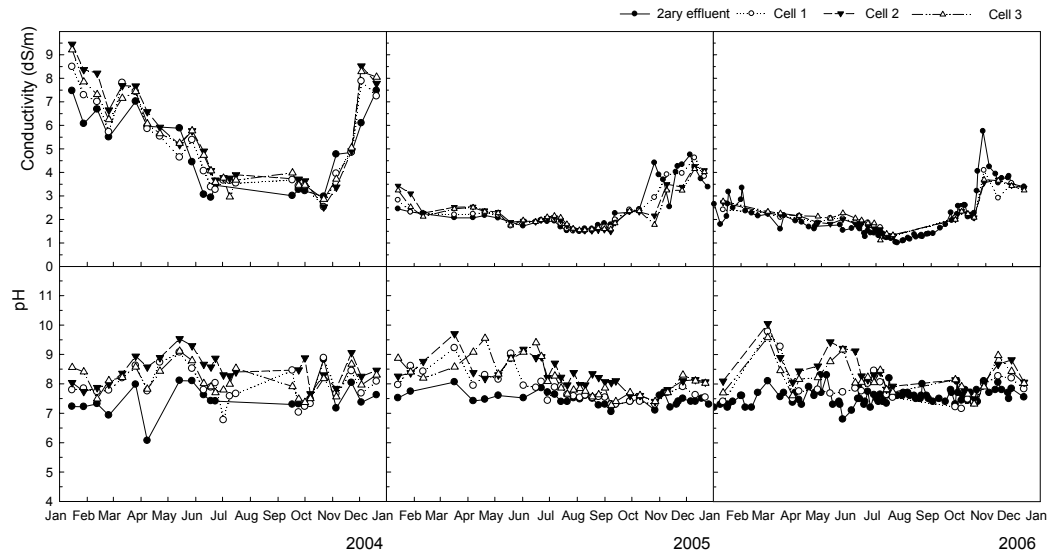

Figure 4. Seasonal variations in conductivity (up) and pH (down) in the WWTP secondary effluent and the cells within the CW from year 2004 to 2006.

Changes in flow affected considerably the water conductivity (Figure 4). Increases in the conductivity out of the peak season appeared concomitantly with an increase in the concentration of sodium chloride, probably reflecting the influence of sea water infiltration to wastewater pipes in the area. In summer such intrusions were counteracted by the high hydraulic loads of WWTP treated effluent. The pH values of the 2ary effluent ranged between 6.8 and 8.8, which generally increased from 0.5 to 2.5 units in the CW cells (6.8-10.1).

In general, the WWTP provided effluents with a highly variable BOD with peaks over 10 mg/L occurring sporadically all trough the year. The most remarkable peak occurred during summer when BOD5 in the effluent raised and reached maximum values of 20 and 32 mg/L the years 2005 and 2006, respectively (Figure 5).

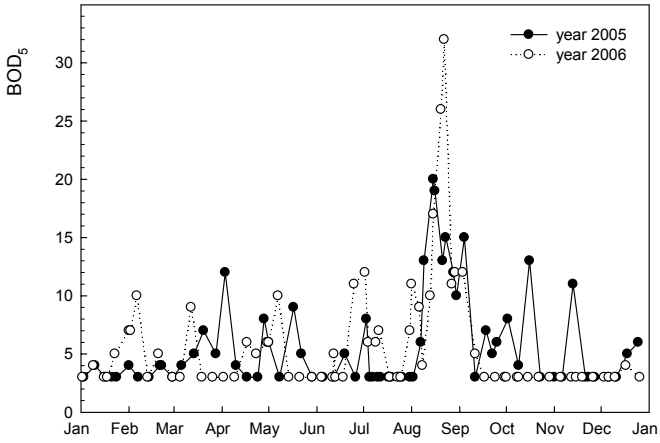

Figure 5. Seasonal variations in the concentration of BOD5 (mg/l) in the WWTP secondary effluent entering the CW during years 2005 and 2006.

Oxygen saturation in the cells showed large differences along time. Minimum values of about 16% saturation were recorded during summer. Generally, the wetland cells showed a good capacity to keep the water oxygenated throughout the year, except for critical summer months, when values of oxygen saturation dropped considerably immediately after the entrance of nutrient peaks in the water. Under critical situations, the aeration capacity of the overlaying water differed significantly in the three treatment cells as derived from oxygen production rates estimated from direct measurements at the inlet and outlet (Figure 6). Oxygen saturation in the water of the CW cells

relative to influent values showed that all treatment cells had periods of deficient aeration. Low performance periods concentrated during summer and were more frequent in cells 1 and 3.

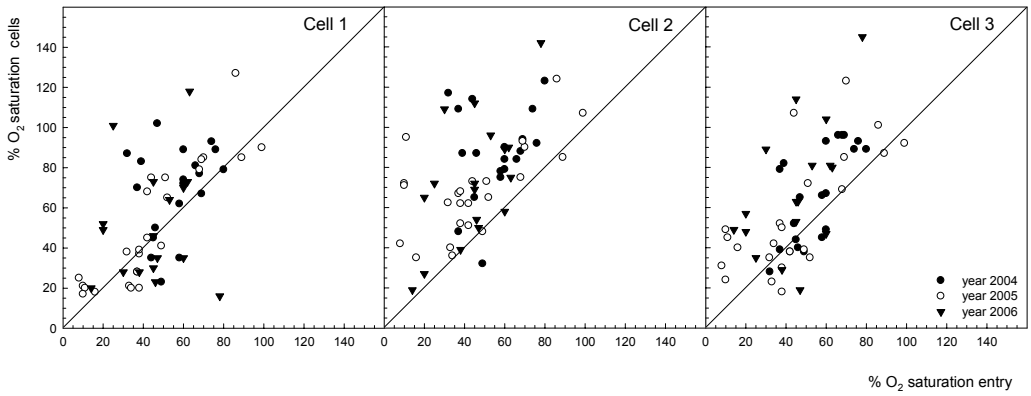

Figure 6. Relationship of the oxygen saturation level (%) between the CW influent and the flow-through water in the three treatment cells from 2004 to 2006. Points below the diagonal line indicate a deficient water oxygenation.

Efficiency in the removal of nutrients

As for total nutrient loads entering the CW, during 2005 the system received an annual load estimated at of 3,300 kg and 9,658 kg of dissolved phosphorous (SRP) and total nitrogen (TN), respectively, whereas in 2006 lower amounts of both (1,993 kg dissolved phosphorous and 6,714 kg total nitrogen) entered the wetland as a result of the continuous operation stops. Minimum loads to the system were recorded during February and were estimated at 173 kg and 114 kg TN, and 65 kg and 52 kg SRP, for 2005 and 2006, respectively. Loads increased roughly a 20 fold during the end of summer; maximum loads were recorded during August 2005 and July 2006 and accounted, respectively, for 1,037 and 536 kg P, and 4,016 and 1,232 kg N, roughly 40% of the total annual load.

The content of soluble phosphorous in the wastewater varied significantly from year 2004 to years 2005 and 2006 (Figure 7). Whereas during 2004 the concentrations of P in the secondary effluent never reached 3 mg/L, during years 2005 and 2006 the WWTP appeared to less efficiently reduce

SRP concentrations and peaks above 6 mg/L SRP were frequently found, specially during summer. Nevertheless, the efficiency of treatment cells was almost constant through the year and buffered phosphorous peaks. Phosphorous concentrations at cells effluent ranged between 0.1 mg/L to maximum values of 3.6 mg/L SRP, and no significant differences were observed between the three cells.

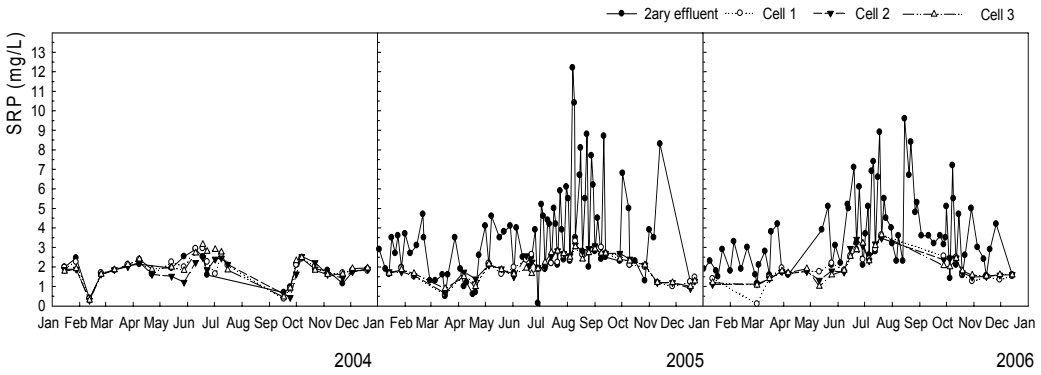

Figure 7. Seasonal variations in the concentration of soluble phosphorous at the inlet and within the cells of the Empuriabrava CW throughout years 2004, 2005 and 2006.

Monthly mean concentrations of total nitrogen measured at the inlet reached values above 20 mg/L during August for all three years studied. Lowest values were recorded during winter of the three years and varied from 1.6 to 2.7 mg/L TN.

Total nitrogen concentration and proportion of the different fractions varied in line with the water input dynamics in the influent. However, despite the similar seasonal pattern, the concentration of NH_4^+ in the wetland influent was significantly higher during the summer of 2005, reaching up to 38.6 mg N/L, in comparison to 26.8 mg N/L in 2006 or 7.2 mg N/L found during year 2004 (Figure 8). Nitrate concentrations at the inlet were negatively correlated with water discharge volumes and higher concentrations were generally found from October to June. Significantly, unusually high nitrate concentrations were reported in the incoming water from January to March 2004, with maximum recorded values above 15 mg N/L. No proven reasoning for this increase in nitrate concentration at the effluent could be provided from the operators at the WWTP.

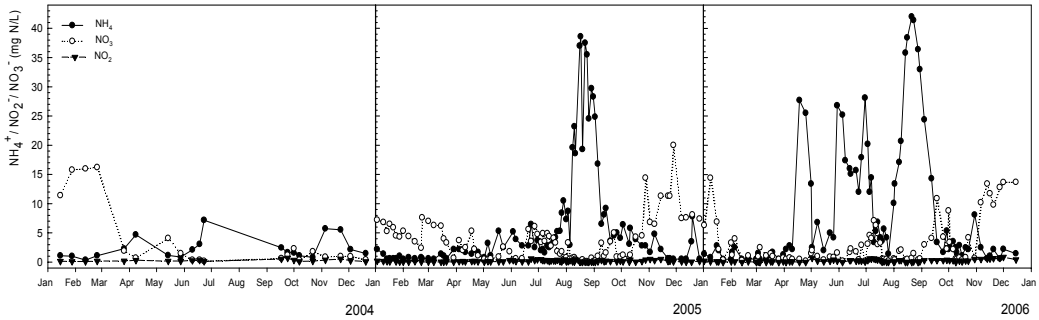

Figure 8. Time variation of NH_4^+ (●) NO_3^- (○) and NO_2- (▼) concentrations at the inlet of the Empuriabrava CW during years 2004, 2005 and 2006.

Ammonia tended to accumulate in wetland cells during summer, showing a deficiency of the oxygenation capacity at the WWTP at high water loads. In all cases, the accumulation of ammonia coincided with a drop of the nitrogen removal rates in the treatment cells. Differences in the performance of the three cells in terms of nitrogen removal were evaluated from the analysis of environmental data at inlet and outlet points and mass balance equations. Water entering cells was independently regulated by means of gravimetric valves, which were checked and modified routinely to obtain similar loads in the three cells. However, no independent measurements of flow were obtained at every treatment cell and values used for calculations were estimated as one third of the total flow entering the wetland.

A large number of processes in a CW rely on the presence and health of the vegetation, among them the control of the velocity of water, which determines to a great extent other mechanisms such as the retention of pollutants (13). Therefore, the differential distribution and density of plants may also affect the water residence time within each treatment cell and cause particular deviations in the hydrodynamics between them.

Years 2005 and 2006 were chosen as representative periods of WWTP and wetland operation to study the performance of cells 1, 2 and 3. It was only during year 2005 that wetlands were continuously in operation despite ammonia concentrations above 7 mg/L. This series of data offered the possibility to evaluate the performance of the CW system along a complete annual time sequence and exemplifies the treatment potential of the wetlands when exposed to severe load and chemical fluctuations without operational intervention. Besides, an estimate of the ammonia

removal potential to set the tolerance limits could be performed. On the contrary, data corresponding to year 2006 were not continuously measured for periods long enough due to repeated flow interruptions that occurred all through the year, which made difficult correct mass balance calculations for many of the months.

Variations in the concentrations of ammonia and nitrate at the effluent of the three independent treatment cells were measured and used as indicative of changes of the nitrogen transformation capacity of the three cells (Figure 9). During year 2005, ammonium tended to increase in the three cells shortly after the concentration peaks in the WWTP effluent. Ammonium increase was faster in cell 1 and occurred shortly after the increase in the WWTP effluent. Maximum concentrations recorded were above 25 mg/L $N\text{-}NH_4^+$ at the beginning of September. Monthly averaged nitrogen removal rates in this cell estimated as mass balance equations yielded negative values for September indicating severe anoxic conditions in the sediment and re-suspension of nutrients within the cell. Cells 2 and 3 showed a similar behaviour although the accumulation of ammonium was not as severe as that recorded in cell 1. The concentration of ammonium increased up to approximately 14 mg/L $N\text{-}NH_4^+$ and peaks were recorded during early September. Compared to cell 1, cells 2 and 3 exhibited a higher nitrate production in the water immediately after the period of high ammonium accumulation, indicating a higher nitrification capacity of the latter cells.

Such behaviour of the three cells was not recorded during year 2006. Repeated flow interruptions and the lack of a continuous data recording made difficult the mass balance calculations for this period. Two peaks of ammonium were recorded in early May and early July in the three treatment cells and coincided after periods of high ammonium concentration at the WWTP effluent. Recorded ammonium concentrations were higher in cells 2 and 3 than cell 1, and reached a peak value of 22.4 mg/L $N\text{-}NH_4^+$ in cell 3.

Global nitrification and denitrification activities may differ considerably in the three cells, resulting in lower nitrogen removal efficiency in treatment Cell number 1 when the system is operated continuously. Unfortunately, the implication of differences in the water load to the three cells as well as other hydrological aspects such as the short-circuiting in some of the cells can not be correctly established due to the lack of data. Besides, as shown above, the three cells vary considerably with respect to the placement and composition of the vegetation, which would have also had a significant impact in the observed changes.

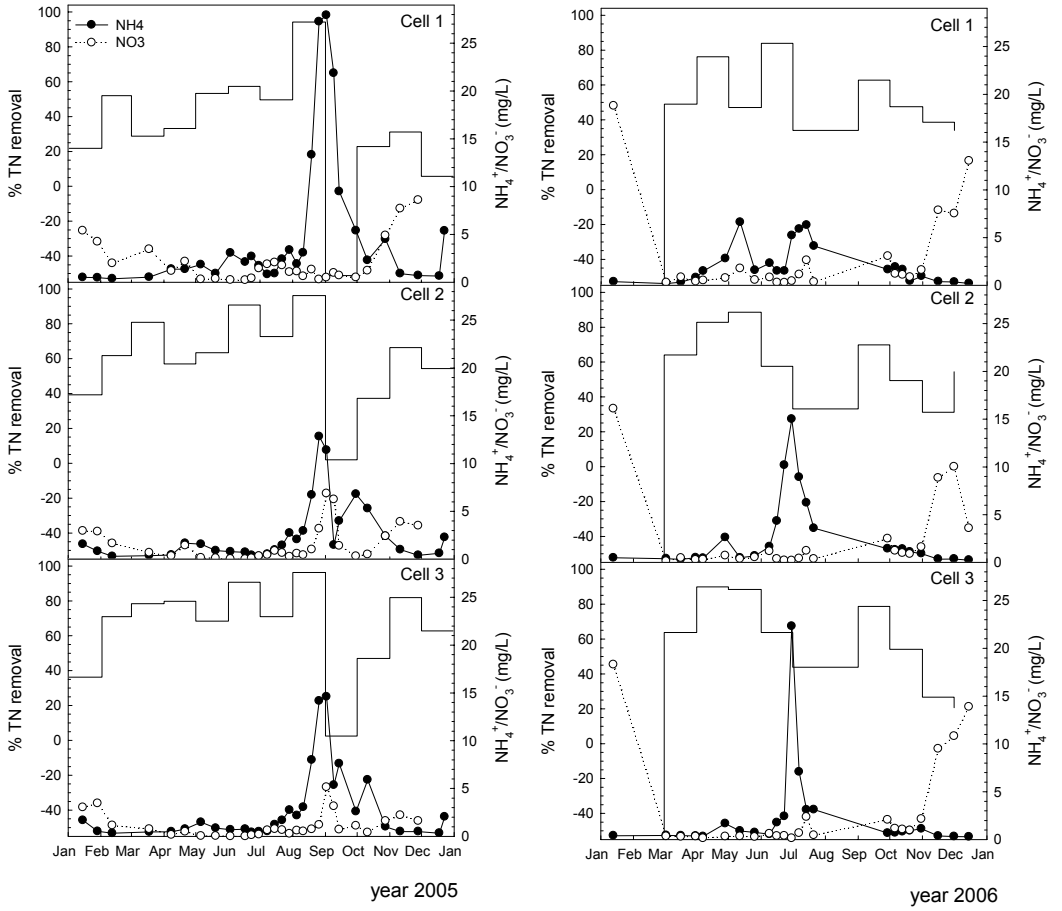

Figure 9. Monthly variations in the total nitrogen removal yield (solid line), residual ammonia (●) and nitrate (o) concentrations at the outlet of treatment cells during years 2005 and 2006.

The presence of oxygen is mandatory for the elimination of ammonia through nitrification (4). During year 2005, the higher accumulation of ammonia in Cell 1 with respect to nitrate and nitrite accumulation in cells 2 and 3, indicated that higher nitrification rates were taking place in the latter cells. The aeration capacity in the treatment cells was calculated as net increases of oxygen concentrations at upstream and downstream measurement points, and was inversely related with residual ammonia in the three cells (Figure 10). The same calculations were done for year 2006 data but the continuous flow interruptions during this period hampered the estimation of the aeration capacity and the conclusions that could be derived.

Plants are known to play a remarkable role for nitrification in constructed wetlands by promoting the development of ammonia oxidizing bacteria in the sediments through the release of oxygen mediated by their root system (2, 9, 11, 12). So far, several studies have documented higher removal rates in wetlands containing plants than in unplanted beds, highlighting the importance of the presence and the relative coverage of macrophytic communities to achieve a better performance, both *in situ* and in experimental systems (5, 8, 10, 15, 16). Presumably, the position of emergent vegetation is a key component that can be easily managed to treat influents of different characteristics. It is generally assumed that better removal efficiencies of NH_4^+-N dominated influents are accomplished in wetlands containing poor vegetated areas upstream (6). This observation held true for 2006 data series, where nitrogen removal rates showed lower efficiencies in cells 2 and 3 when ammonia accumulation occurred during May and water retention times within the treatment cells ranged between 5 and 6 days. Ammonia accumulation occurred at periods of lower water retention times (August 2005, HRT_{min} = 2 days) resulted in lower performances in cell 1.

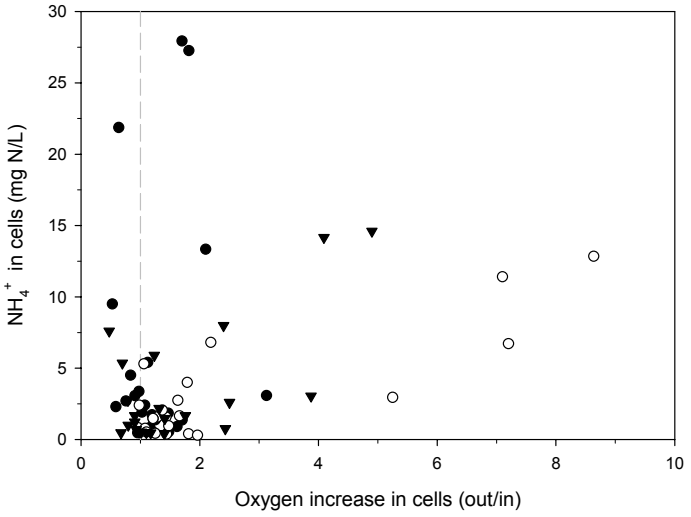

Figure 10. Excess ammonia concentration at the outlet of treatment Cell 1 (●), 2 (○) and 3 (▼) in relation to oxygen increase ratios during year 2005.

The plant species composition, either as mixed vegetation communities or monocultures, has also been extensively shown to influence total nitrogen removal rates in CWs (1, 7, 15). To exemplify, Zhu and Sikora (16) could confirm that nitrogen removal was achieved more efficiently in

wetlands planted with *Phragmites* sp. than in those containing *Typha* sp. Such plant to plant variations have been explained by differences in root development, sediment penetration depth and amount of oxygen released through vegetal tissues (3).

Besides the plant coverage, the plant species composition also varied significantly from cell to cell. However, according to the difference in the removal yields, it seems that the location of vegetation stands within the treatment cells would have a stronger influence on the observed results compared to species composition. Cells 2 and 3, both have higher plant densities upstream the treatment cell, although the former is mainly covered with cattail and the latter with common reed.

Final considerations

The Empuriabrava CW has experienced many variations in its performance, affecting mainly the water load from year 2004 to 2006. Initially developed as a nitrate polishing system, the extraordinarily high ammonia loads have given rise to numerous operational incidents, mostly during summer, which have forced the application of several modifications in the operational parameters all throughout this period, as measures to establish an appropriate operational set up adjusted to real demands.

As shown previously, problems in the CW efficiency are inherent to high ammonia loads and development of anoxia in the treatment cells. Nitrification activity at the WWTP was stated as the critical factor to provide water of the desired quality for flooding the Wetland area. Modifications of the WWTP were designed to improve aeration capacity and favour the supply of a nitrified effluent. In order to cope with this need, and due to the lack of unpredictable errors in the scale-up criteria used for the actual two Carrousel oxidation ditches that can not account for the high nutrient loads in summer, a third line has been incorporated to guarantee a completely nitrified effluent. Since its implementation and fully operative (late 2006), the third line in the WWTP provides effluents with a low content of NH_4^+, which are easily treated in the CW and purified to satisfactory standards regardless of changes in the water loads that occur during year.

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3.3. CHANGES IN THE COMPOSITION OF NIRS GENES IN THE RHIZOSPHERE OF TYPHA SP. AND PHRAGMITES AUSTRALIS IN A CONSTRUCTED WETLAND ASSESSED BY T-RFLP

Changes in the composition of *nir*S genes in the rhizosphere of *Typha sp.* and *Phragmites australis* in a constructed wetland assessed by T-RFLP

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ABSTRACT

The role of plants in the community structure of denitrifiers of a FWS-CW was investigated over two consecutive years by analysing both areas covered with cattail (*Typha* sp.) and common reed (*Phragmites australis*) and sediments without vegetation. Multidimensional scale plots (MDS) of T-RFLP fingerprints of the heme cd_1 containing nitrite reductase coding gene (*nir*S) revealed inter-annual variations as the main factor determining shifts in the bacterial community with a stress factor lower than 0.15. An analysis of the variance (ANOSIM) test confirmed time as significant factor explaining sample ordination of both bare sediments and rhizosphere samples. The observed differences were related to the growth stage of plants. Differences between plant species were mainly related to minor T-RFLP peaks, accounting individually for less than 2% of the relative abundance and could not be resolved with the ANOSIM test. An ordination method based on principal component analysis (PCA) with log transformed T-RFLP data revealed that a minimum of three components were needed to cover more than 60% of the variance observed. PC1, covered 31% of the variance and showed a strong time component. PC2 and PC3, each contributing to 14% of the variance, showed a sample ordination according to plant species. Most relevant variables, T-RF fragments, could be undoubtedly assigned to *nirS* gene sequences in the database through comparison with *in silico* digestions. Members of distinct phylogenetic groups are shown to change in the rhizosphere of common reed and cattail according to plant growth. The obtained results indicate a significant change in the population of denitrifiers which may correlate to changes in the nitrate removal activity of the constructed wetland.

INTRODUCTION

Constructed wetlands (CW) are nowadays recognised as feasible alternatives for water purification. They are widespread as wastewater treating methods for diffuse waste coming from agricultural, industrial and municipal activities and provide final polishing alternatives for promoting water reuse (9, 28) Complete nitrogen removal in constructed wetlands receiving mostly ammonia-rich inflow is achieved by alternate nitrification and denitrification steps (16, 37), being the former the critical step (17). Both processes are meant to occur either in subsurface flow (SSF) or free flowing water surface wetlands (FWS-CW) and are basically microbial activities. However, in treatment wetlands coupled to conventional WWTP the final polishing of nitrogen residues often only requires a nitrate reduction step, mainly carried out by denitrifying bacteria. Nitrates in the water are sequentially denitrified under low oxygen concentrations in a four-step pathway to completely reduced N_2. First, nitrate is converted to nitrite, which is further transformed to nitric oxide (NO) and subsequently to nitrous oxide (N_2O) and N_2. True denitrifiers are generally heterotrophic bacteria, although denitrification can also be attributed to some *Archaea* and fungi (39). Nitrite reduction to nitric oxide is the critical step that distinguishes true denitrifiers from nitrate-respiring bacteria [zumft62]. Nitrite reductases (nir) are the enzymes responsible for this chemical step, and can be found among denitrifiers in two structurally but physiologically and functionally equivalent versions (13, 39). Cu-nir contains copper and is encoded by the *nirK* gene, while cd_1-nir contains groups heme and heme d_1, and is encoded by the *nirS* gene.

Vegetation participates in numerous functions in CWs, including both physical actions and enhancing some metabolic reactions leading to nutrient removal by other organisms basically bacteria (33). Of particular interest for nitrogen removal, plant roots may increase small scale heterogeneity in the sediment surface by the exudation of nutrients, which will promote the alternative growth of heterotrophic bacteria and, providing adequate anoxic conditions, will stimulate denitrification. On the contrary, the release of oxygen at the root surface will be a key issue for the activity of ammonia oxidizing microorganisms, stimulating nitrification (27, 28).

So far, several studies have documented higher removal rates in wetlands containing plants than in unplanted beds, which have been proved both *in situ* and in experimental systems at laboratory or pilot scales (15, 18, 22, 35, 38). Moreover, some research has been conducted to highlight some of the characteristics that plant species may have to support different treatment processes (2, 11, 38). Nevertheless, how microbial communities and their respective activities are governed by wetland vegetation is still poorly understood (34).

Denitrification is regarded as a selective trait for the colonization of the rhizosphere surface as has been derived from incubation experiments with non-denitrifying mutants or from bacterial density measurements at the root surface of certain plants (12, 36). Although surveys based on the heme cd_1 containing nitrite reductase harbouring bacteria offer a restricted view of denitrifiers diversity, the present study focused exclusively on the gene coding for this enzyme since we had failed to detect copper-containing nitrite reductase (NirK) harbouring denitrifiers in previous attempts (30). Other authors have pointed out the apparent mutual exclusivity of either type of nitrite reductase in several soil and aquatic environments (6, 23, 25, 31).

To date only a few studies are available about the possible variations in denitrifying microbial

communities implicit to plant distribution in wetlands. In FWS-CW shifts in denitrifying communities were coupled to plant distribution and correlated to changing water retention times and nutrient input (19). Similarly, the molecular survey of heme cd_1-containing nitrite reductase coding gene (*nirS*) yielded a completely different pattern and increased richness of *nir*S genotypes in sediments of a freshwater wetland occupied by an invasive cattail species (*Typha* x *glauca*), indicating plant specific changes in the diversity of denitrifiers (1). In terrestrial ecosystems such as agricultural soils the plant species seemed to determine the occurrence of characteristic denitrifying bacteria, with an additional temporal effect related to seasonal conditions or plant development (7).

The goal of our study was to investigate to what extent the presence of macrophytic vegetation stands affected the structure of the denitrifying communities in the treatment cells of the Empuriabrava FWS-CW. The Empuriabrava WWTP (Costa Brava, Spain) is complemented with a free water system constructed wetland consisting of three parallel plug-flow cells of comparable size and surface that receive similar nutrient loads and initially designed as final polishing step to provide water of good environmental quality to flood a nearby located natural reserve. The present study focuses on the evaluation of *nirS* gene in the rhizospheres of the main plant species *Phragmites australis* and *Typha* sp. The abundance and coverage of stands of both plant species are easily modified and increasing the knowledge of associated microbial communities is fundamental for management purposes.

MATERIALS AND METHODS

Sampling procedure

Samples of the bulk sediment of a non-vegetated area (S samples), and the rhizosphere of dense vegetated areas of *Phragmites australis* (PH samples) and *Typha* sp. (TY samples) were collected in May 2004 and July 2005, from sampling stations located at different positions within the third cell (Figure 1).

Data on the physicochemical conditions of the wetlands influent and flow-through water in the treatment cells was collected every 15 days and kindly provided by the CW personnel (Table 1).

A 7 cm diameter plexiglas tube mounted in a manual core sampler was used for bare sediment collection. Roots and root fragments were carefully removed by eye inspection from sediment samples, which were after manually homogenized using a sterile spatula. Rhizosphere samples were obtained after harvesting a sufficient number of plant shoots and removing the loose sediment portion from roots washing them twice with sterile isotonic solution. Roots were selected and cut in approximately 0.5 cm pieces and homogenized. Three replicates of either bare sediments or rhizospheres were obtained for year 2004. The replicate number was increased to five for year 2005 since some of the samples failed to provide good quality nucleic acid extractions. In all cases, one gram aliquots of all replicate samples were frozen at -20ºC for further molecular analyses.

DNA Extraction and PCR amplification of nir*S genes*

Nucleic acids were extracted using the MOBIO Ultraclean Soil DNA kit (MO BIO Laboratories, Inc.) following the manufacturer instructions. Quality of DNA extracts was checked on agarose gels and concentration was measured in a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington, DE, USA).

Amplification of *nirS* gene fragments was performed in a Geneamp® PCR system 9700 (Applied Biosystems ABI, Foster City, CA),

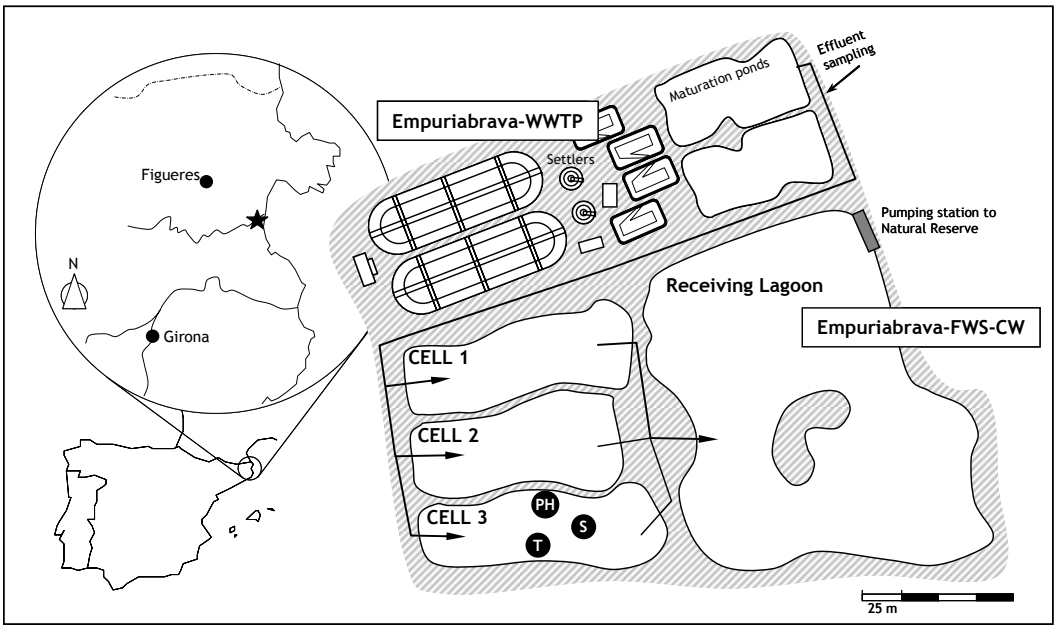

Figure 1. Empuriabrava WWTP and adjacent free flowing surface constructed wetlands system (CWS). Sampling locations on the CWs are indicated. PH: *Phragmites australis* community area, TY: *Typha* sp. community area, S: bulk sediment area.

with the primer pair nirS1F:nirS6R (4) and PCR conditions as described previously (30). nirS1F primer was labelled with 6'-carboxyfluorescein (6-FAM) for T-RFLP analyses.

T-RFLP Analysis of amplified nir*S genes fragments*

The products of two positive PCR reactions for each sample were pooled and concentrated by a conventional ethanol precipitation protocol (5). After concentration, DNA was rehydrated in 20-30 μL of sterile distilled H_2O. The DNA was then loaded onto a 0.8% (wt/v) agarose gel and electrophoresed for 45 minutes at 80 mV. Bands of size corresponding to the *nirS* amplicon (~ 900 bp) were excised, cleaned and eluted in a total volume of 20μL using the Qiaquick Gel Extraction Kit (Qiagen).

Total digestion of the *nirS* gene purified PCR products (100 ng) was performed overnight at 37ºC in 24 μL reactions containing 10 U of restriction endonuclease, 2.5 μL of 10X buffer provided with the enzyme and 0.5 μL of 0.1% BSA. Sample triplicates were cleaved with different endonucleases, *Alu*I, *Msp*I and *Hha*I (Takara BIO Inc.). Reactions were precipitated with ethanol. 3 μL of the resuspended pellet were mixed with 20 μL of formamide and 0.1 μL of 500 bp *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (TAMRA GS500, Applied Biosystems) added as internal standard. After DNA denaturation, consisting on 5 min at 94ºC, and immediate chilling on ice for 5 min, the DNA was loaded onto the 36 cm long polymer capillary (POP4, Applied Biosystems) of an ABiprism 310 Genetic Analyzer. Electrophoresis was run for 30 min at 60 ºC, with limits of 15 kV and 7 mA and the T-RFs length was automatically analysed by the Genescan Analysis Software Version 3.1 (Applied Biosystems) by comparison with the

Table 1. Main physicochemical parameters of the water at the inlet and effluent of the treatment cells for May 2004 and July 2005. Values are given as the monthly averages ± SD. Data were provided by the personnel at the WWTP.

	May2004				**July 2005**			
	Inlet	**Cell 1**	**Cell 2**	**Cell 3**	**Inlet**	**Cell 1**	**Cell 2**	**Cell 3**
pH	8.1 ± 0.0	8.7 ± 0.3	9.4 ± 0.1	8.9 ± 0.2	7.4 ± 0.1	7.7 ± 0.1	8.3 ± 0.3	7.8 ± 0.2
Conductivity (dS/m)	5.1 ± 1.0	5.0 ± 0.5	5.4 ± 0.4	5.5 ± 0.3	1.6 ± 0.1	1.7 ± 0.2	1.8 ± 0.2	1.9 ± 0.2
Temperature (ºC)	19.9 ± 3.3	19.9 ± 2.9	19.8 ± 3.1	19.6 ± 3.3	25.2 ± 2.2	25.0 ± 2.6	25.1 ± 2.4	25.1 ± 2.1
% O2	77 ± 4	86 ± 9	116 ± 9	89 ± 0	47 ± 13	57 ± 19	62 ± 9	43 ± 17
N-NO2 (mg/l)	0.17 ± 0.00	0.07 ± 0.07	0.07 ± 0.04	0.09 ± 0.04	0.27 ± 0.10	0.26 ± 0.11	0.11 ± 0.05	0.10 ± 0.02
N-NO3 (mg/l)	2.77 ± 1.83	0.80 ± 0.35	0.55 ± 0.41	0.94 ± 0.82	3.06 ± 1.44	1.69 ± 0.43	0.55 ± 0.25	0.57 ± 0.21
N-NH4 (mg/l)	1.01± 0.20	0.62 ± 0.31	0.42 ± 0.02	0.44 ± 0.07	4.26 ± 2.72	1.87 ± 1.23	1.41 ± 0.95	1.53 ± 0.93
TIN (mg/l)	3.96 ± 2.02	1.50 ± 0.73	1.05 ± 0.48	1.48 ± 0.94	7.60 ± 1.72	3.83 ± 0.97	2.07 ± 0.89	2.21 ± 0.79
SRP (mg/l)	1.94 ± 0.42	2.24 ± 0.17	1.52 ± 0.21	1.98 ± 0.11	3.83 ± 1.35	2.22 ± 0.26	2.50 ± 0.38	2.61 ± 0.25

internal standard. Peaks with values below 50 fluorescence units and fragments shorter than 50 bp or larger than 600 bp were discarded from the analysis. Profiles were inspected for overlapping or skimmed peaks, which were automatically split by modifying software peak detection specifications. T-RF profiles were exported and peak areas normalized to total area. The relative area was used as an estimation of the abundance of each terminal restriction fragment within a sample (3).

Statistical analysis

The effects of vegetation and plant species, as well as inter annual variations, on the T-RF profiles were explored by hierarchical cluster analysis using the PRIMER 6 software (Primer-E Ltd.). Only those peaks contributing at least to 1% to the total area were selected to calculate a similarity matrix using the Bray Curtis coefficient, which was further ordinated to generate a non metric multidimensional scaling (MDS) plot. Stress values calculated according to Kruskal (20) were used as estimation of the "goodness of fit" of the ordination analysis (8). An ANOSIM test with 10,000 permutations was used to assess the significance of differences in the community structure between data groups created according to the factors vegetation and year.

Principal Component Analysis (PCA) was applied alternatively to the same T-RFLP data using the application of the PRIMER 6 software and setting a maximum extraction of 3 PC, to extract main factors involved in sample distribution. For that purpose the T-RFLP data was log transformed and only rhizosphere samples were analysed, in order to independently evaluate the influence of a singular plant species on the T-RFLP profiles of *nirS* harbouring denitrifiers. The most relevant variables, that is T-RFLP fragments with higher contribution to explain the ordination of samples were found according to the eigenvector values.

In silico T-RFLP analysis of nir*S clone sequences*

The distinct terminal restriction fragments (T-RFs) of denitrifiers obtained with direct T-RFLP analysis of sediment and rhizosphere samples were compared to the theoretical fragment sizes of a collection of *nir*S gene sequences obtained from sediments of the Empuriabrava CW (30). The partial sequences of the *nirS* gene obtained from 166 clones were digested *in silico* with the three endonucleases

used in this study, using the Bioedit Sequence Alignment Editor Version 7.0.4.1 software (14).

RESULTS AND DISCUSSION

T-RFLP profiling of nirS *gene fragments*

A total of 52, 36 and 44 distinct terminal restriction fragments (T-RFs) were distinguished from the digestions with *Alu*I, *Msp*I and *Hha*I, respectively. *Alu*I profiles were mostly dominated by few fragments, with 181 bp and 338 bp being the most abundant. In turn, *Msp*I digestions yielded more evenly distributed T-RFs within every sample, with 95, 97, 137, 139 or 370 bp being the most common sizes. *Hha*I generated fragments were shared by all samples with only some occasional specific peaks (Figure 2).

From 2004 to 2005 the composition of T-RFLP profiles generally increased in complexity. Fragments of 51 bp, 53 bp, 70 bp (*Alu*I), 139 bp (*Msp*I), 345 bp and 382 bp (*Hha*I) were found in higher proportion in rhizospheres of 2004, whereas others such as 337 bp (*Alu*I), 167 bp (*Msp*I) became more abundant the following year. The behaviour of several T-RFs also varied in association with the presence of vegetation. Relative abundances of 139 bp (*Msp*I) and 108 bp (*Hha*I) fragments were high in 2004 rhizosphere samples and decreased noticeably the following year. On the contrary, the same fragments were kept at similar abundances in bare sediments. Such opposed changes in the abundance of T-RF between profiles of sediments and rhizospheres on both sampling dates would be indicative of alternative responses of the corresponding denitrifying populations in a determined environment.

Finally, the shifts in abundance of other T-RFs seemed to occur in relation to plant species. An *Alu*I generated T-RF of 181 bp was found in a constant proportion in the profiles of all samples, regardless the rhizosphere of *Typha sp*. during May 2004, where it was found at much lower relative abundances. Plant specific shifts in relative abundances of fragments were also detected for many other examples showing in some cases opposite trends (i.e. 79 bp T-RF obtained with *Msp*I or the HhaI generated 270 bp fragment).

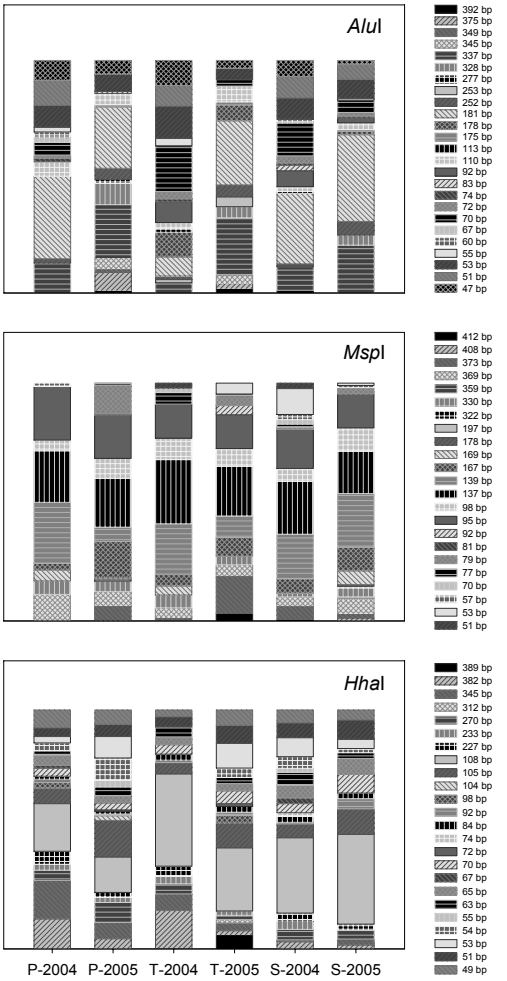

Figure 2. Relative abundances of T-RFLP fragments obtained with digestion of PCR amplified *nirS* genes with endonucleases *Alu*I, *Msp*I and *Hha*I. P- rhizosphere of *Phragmites australis*. T- rhizosphere of *Typha sp*. S- bare sediments. Samples corresponding to years 2004 and 2005 are indicated.

Fragments of plant specific sizes were found as minor peaks, often contributing independently to less than 2% of the total abundance (Table 2). Samples retrieved during year 2005, yielded a relatively higher number of T-RFs especially when *Alu*I was used as a restriction endonuclease. Moreover, the abundance of plant species specific peaks was higher in these samples suggesting an effect of the rhizosphere in the development of certain denitrifying populations.

Multi dimensional scaling of T-RFLP profiles

Despite the differences observed in the richness of retrieved *nirS* gene T-RF fragments, inter-annual heterogeneity was shown to be the keynote variable in sample distribution as derived from MDS plots (Figure 3) although stress values indicated a poor definition. The MDS analysis combined with the ANOSIM test has been proven a useful tool to analyze T-RFLP data and used thoroughly (29). In the MDS analysis the stress value is used as a measure of the goodness of fit of the data. It is generally assumed that a stress value greater than 0.2 indicates that the plot is close to random and no differences are supposed to exist between user defined classes. A plot with stress values less than 0.2 can be considered a useful bi-dimensional representation and only values less than 0.1 would correspond to an ideal ordination (8). In our case, the stress value was 0.15, which can certainly be taken as a quite reliable picture of sample differences although not totally exempt from misinterpretations.

According to the MDS plot, even though the rhizospheres appeared to be more closely related to sediments within the same sampling date than to rhizospheres from the consecutive year, a distinction between groups of replicates of bare sediments, *Typha* sp. and *Phragmites australis* rhizospheres could still be appreciated, suggesting the presence of specific denitrifying communities coupled to each kind of sample. The relationships between sample types were evaluated with an analysis of similarity (ANOSIM) test. ANOSIM tests the null hypothesis that the average rank similarity between samples within a group is the same as the average rank similarity between samples between groups. ANOSIM is based on the rank similarities between samples in the similarity matrix and produces a test statistic, R, which can range from -1 to 1. An R value greater than zero indicates the presence of higher dissimilarities for samples between groups, whereas an R value of 0 indicates that the null

Table 1. Distinct T-RF fragments of *nirS* genes for each subset of samples. Richness is presented as the mean (± standard error of the mean) number of terminal restriction fragments produced by T-RFLP analysis using *nirS* gene primers. Zone and time specific fragments within the samples are indicated

	***Alu*I**		***Msp*I**		***Hha*I**	
	N	**Specific T-RF fragments (bp)**	**N**	**Specific T-RF fragments (bp)**	**N**	**Specific T-RF fragments (bp)**
May 2004	14.1 ± 2.5	67 / 72	14.6 ± 3.1	57/ 70 / 51	22.5 ± 5.9	
Typha sp. (n=2)	13.0 ± 2.8		14.0 ± 2.8		17.5 ± 4.9	
P. australis (n=2)	13.5 ± 0.7		16.5 ± 3.5		17.5 ± 0.7	
Sediment (n=4)	15.0 ± 3.1	153	14.0 ± 3.5		27.5 ± 2.5	
July 2005	18.6 ± 3.1	175 / 307 / 375 / 439	15 ± 2.5	81 / 178 / 353 / 359	20.7 ± 3.9	126 / 151
Typha sp. (n=4)	18.2 ± 2.2	345 / 277	13.5 ± 3.7	193 / 412	20.7 ± 4.5	435
P. australis (n=5)	18.4 ± 2.9	279 / 324	16.2 ± 1.6	197 / 234	20.2 ± 4.6	
Sediment (n=4)	19.5 ± 4.6	153	15.2 ± 1.2		21.2 ± 3.5	

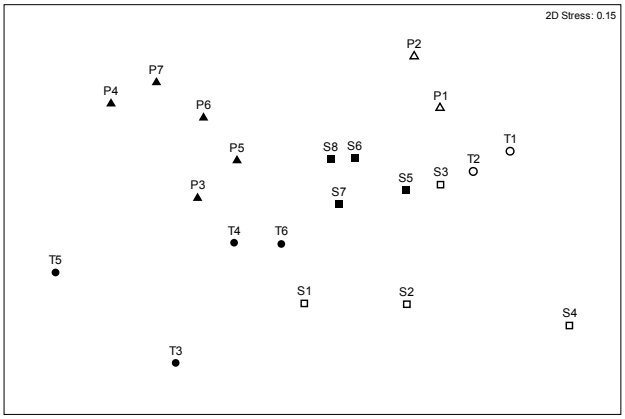

Figure 3. MDS plot based on Bray-Curtis similarity matrices of T-RFLP patterns for nirS gene fragments digested with *Alu*I, *Msp*I and *Hha*I from bare sediments (S)(□, ■) and rhizospheres of reed (P)(▲, Δ) and cattail (T)(○, ●). Open symbols refer to samples collected on May 2004 and filled symbols to July 2005.

hypothesis is true (29). Finally, a level of significance (p value) is also produced. Applying the test in a 2 way crossed analysis with 10,000 permutations rendered R and p values that confirmed the existence of significant differences in both time (R = 0.893, p = 0.01) and vegetation compared to bulk sediment across all year groups (R = 0.45, p = 0.02). However, the ANOSIM test did not yield differences of individual plant species.

Plant driving changes on denitrifying bacteria composition

A deeper insight in the differences between rhizosphere samples of different plant species was obtained using principal component analysis (PCA) as a data reduction procedure. Fluorescence data obtained from T-RFLP were log transformed to ensure a normal distribution prior to ordination using PCA (21). Although some doubts exist about data transformation and the reliability of the outcome of PCA ordinations when T-RFLP data is used (10, 26), this method was chosen to test which were the variables, i.e. T-RF fragments, showing a greater contribution to sample ordination.

A minimum of three components were needed to explain above the 60% of the variance found in rhizosphere samples (Figure 4). PCA plots again emphasized the time variation of samples over the origin of samples. First component (PC1) explained up to 31.1% of the observed variance and was clearly indicative of sampling time. The separation of *Typha sp*. and *Phragmites australis* was observed along the axis of the second component (PC2), which contributed with a 14.8% of the variance. PC3, contributed with an additional 13.7% of the variance and stressed the observed variation between rhizosphere samples. Nevertheless, the interpretation of PCA plots is rather subjective compared to a more quantitative approach such as the previously performed similarity analysis with the ANOSIM test, which could not confirm the presence of significant variations between rhizosphere samples.

The values of the eigenvectors of PC1 and PC2 provided by the PCA analysis were used to quantify the contribution of each T-RF fragment. The influence of the overall set of variables was quite homogenous indicating the contribution of a large set of T-RFs to the values of PC1 and PC2. Higher values of the eigenvectors explaining PC1 were calculated for the terminal restriction fragments of 79 bp and 373 (*Msp*I) and 382 bp (*Hha*I), the latter

having a negative value. The most significant variables involved in PC2 were 53 bp (*Msp*I) and 270 bp (*Hha*I), having opposite contributions. The T-RFs highly contributing to PC1 and PC2 derive basically from T-RFLP analyses performed with the endonucleases

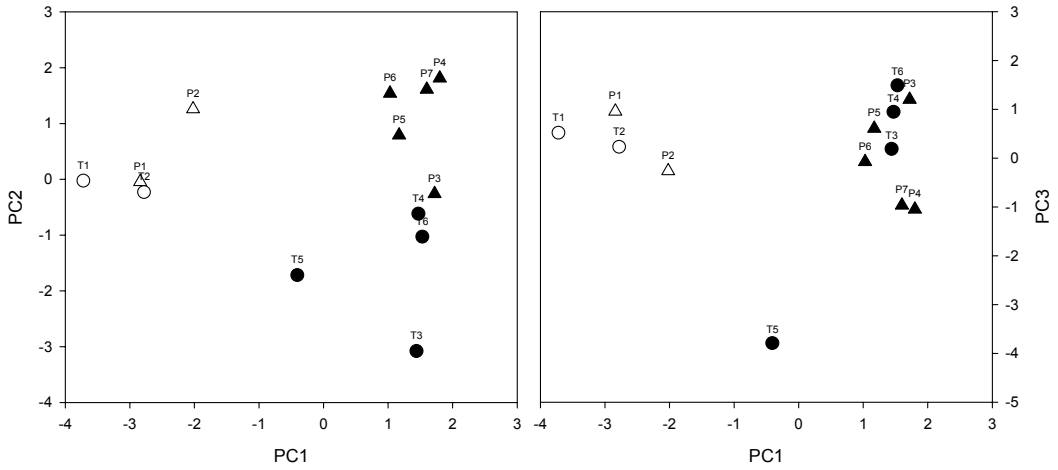

Figure 4. PCA ordination plot of *nirS* gene T-RFLP patterns of rhizosphere samples collected during May 2004 and July 2005. A logarithmic transformation was used to normalize the T-RFLP data. P- *Phragmites australis* rhizosphere (Δ, ▲). T- *Typha latifolia* rhizosphere (○, ●). Open symbols refer to samples collected on May 2004 and filled symbols to July 2005.

*Msp*I and *Hha*I. Therefore, these two enzymes seem to possess a greater resolution capacity when compared with *Alu*I.

According to T-RFLP data, a high diversity of NirS harbouring bacteria is supposed to occur in both the sediment and the rhizosphere, although few fragments dominated all situations considered. The T-RFLP approach discriminated between sediments and rhizospheres but was not conclusive in identifying plant species specific shifts on denitrifiers by any of the ordination methods used. Conversely, the statistical analysis of T-RFLP groups stressed the impact of annual over plant specific variations. Actually, a large set of environmental factors co-vary from year to year, including meteorological conditions, communities in the rhizospheres vary also from year to year, although the distance between particular plant species groups is kept. The results suggest that denitrifying communities in sediments follow divergent dynamics from the plant associated denitrifiers and thus the differential behaviour through the two periods indicates that the variations that occur in the community structure are not only time dependent but also connected to the specific site in which bacterial populations develop This is in agreement with other studies and implies an additive effect of other factors, probably related to plant physiology and development stage, which actively participate in the establishment of characteristic denitrifiers (7, 32).

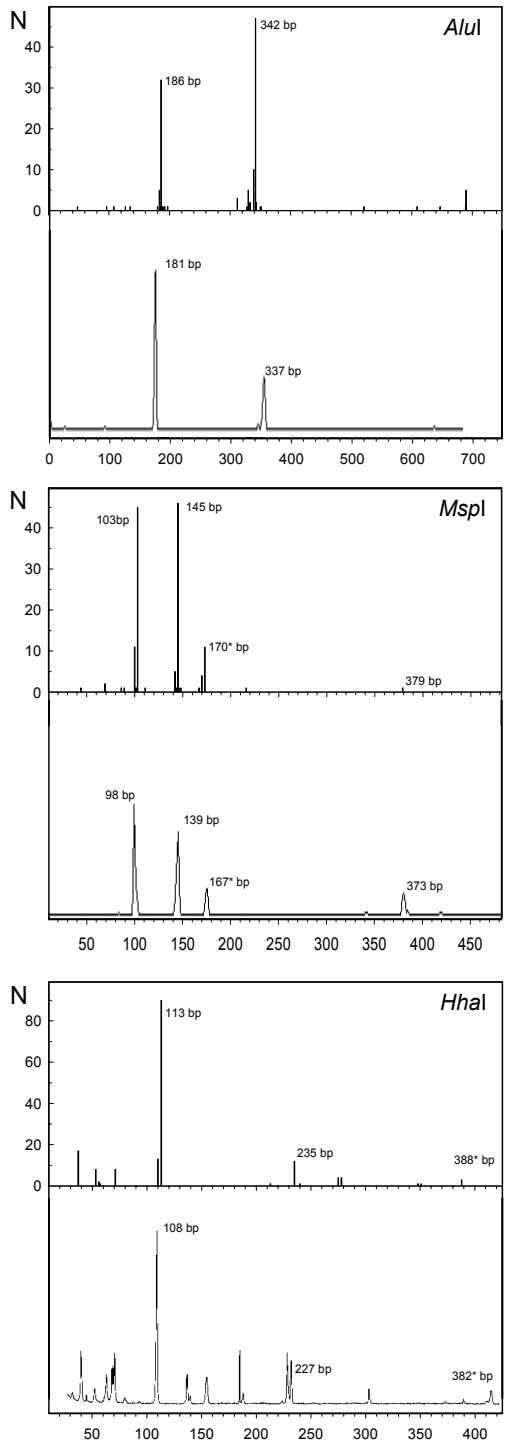

Figure 5. *In silico* restrictions of 166 *nirS* partial sequences (top) and experimentally obtained T-RFLP profiles (down) with the endonucleases *Alu*I, *Msp*I and *Hha*I. Peaks for which correspondences could be established appear with the corresponding size (bp). Asterisks show restriction fragment exclusively found in Group II clones (30).

The comparison of T-RFLP profiles of samples coincided with the graphical representation of *in silico* digestion of partial *nir*S gene sequences (Figure 5). Sequences were obtained from bare sediments of the Empuriabrava CWs and deposited in the GenBank database under the accession numbers EF558372 to EF558536 (30). All retrieved *nirS* partial sequences in this former study clustered in two separate major groups, which responded to changes in environmental conditions. Considering the peaks with higher relative abundances, it was possible to establish the correspondences of fragment lengths to *in silico* fragments and some of the present microorganisms in sediment samples could be identified accordingly. Generally, experimental T-RFs matched with the length of theoretical ones with differences of 3 to 6 bp. Theoretical T-RFs of 388 for *Hha*I and 379 for *Msp*I matched with 382 bp (*Hha*I) and 373 bp (*Msp*I) fragments, and contributed significantly to PC1 variability. In silico fragment 379 (*Msp*I) was found exclusively in sequences corresponding to Group I, whereas 388 (*Hha*I) was exclusive for Group II clones. The same correspondence could be made with T-RF 139 bp and 167 bp of *Msp*I, assigned to theoretical fragments of 145 and 170 bp and found in clones belonging to groups I and II, respectively. From the obtained results and the possible assignations, it seems clear that PC1 ordinates rhizosphere samples according to relative changes in the proportion of Group I and II clones, although no distinction of defined subgroups can be done.

Although no direct pattern associated with increased proportion of the T-RFs characteristic

for a clone group could be found to explain the differences between samples, shifts in abundance and presence of these T-RFs in the profiles reflect how populations of denitrifiers are selected depending on the site they inhabit or the prevalent environmental conditions. The presence of plants indeed played a role in determining microbial diversity, which may be influenced by the developing stage of the plant, since little changes in the physical and chemical composition of water overlaying sediment were recorded. Moreover, an interaction of time and plants was reflected by MDS and PCA plots and confirmed by the R values and p significance values of the ANOSIM test. Thus, rhizospheres may constitute a particular environment, where a complex interaction of variables connected in turn with time variation determines in the last term the final community.

Although changes in the composition of the denitrifying bacteria in relation to plant species could be detected to some extent, it remains to be elucidated what are the implications of these structural differences on the function of denitrifying communities. Significantly higher potential denitrification activities in terms of dry weight have been reported for the rhizosphere of *Typha sp.* and *Phragmites australis* when compared to bare sediments, but differences are not maintained when samples from different seasons are compared within the same year (Ruiz-Rueda et al FEMS submitted). Moreover, variations in the potential denitrification activity of denitrifying communities may also occur in different years, or at different locations over the wetland surface (19, 24). None of the previous studies established an experimental relationship between changes in the community structure of denitrifying bacteria and the measured activities.

The understanding of the connection between community structure and function is therefore an outstanding question that certainly needs to be addressed to better assess the possible effects of microbial reorganisations in terms of function of the constructed wetlands.

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3.4. STRUCTURE AND FUNCTION OF DENITRIFYING AND NITRIFYING BACTERIAL COMMUNITIES IN RELATION TO THE PLANT SPECIES IN A CONSTRUCTED WETLAND

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Abstract

The community structure and potential activities of nitrifying and denitrifying bacteria were studied in the rhizosphere of Typha latifolia and Phragmites australis present in a free water system constructed wetland (CW). Potential nitrate reduction and nitrification activities were shown to be significantly higher in the rhizosphere when compared with the nonvegetated sediment. Higher rates were generally obtained for P. australis. The community structure of denitrifying bacteria in the rhizosphere differed from that found at the bulk sediment, as revealed by PCR-denaturing gradient gel electrophoresis (DGGE) of the nitrous oxide reductase encoding gene nosZ. Results also show a greater nosZ genotype diversification and suggest a plant species effect in rhizosphere samples obtained during events of low hydraulic retention times. Ammonia-oxidizing communities were less complex on the basis of PCR-DGGE analysis of the 16S rRNA gene. Retrieved sequences were all related to Nitrosomonas marina and Nitrosomonas ureae, being both present in rhizosphere and bulk sediment regardless of environmental changes. The results demonstrate the effect of vegetation on the functioning and structure of bacterial communities involved in the removal of nitrogen in the treatment cells of a CW and point to the use of vegetation coverage to promote nitrification or denitrification in particular areas.

Keywords

denitrifying bacteria; vegetation; rhizosphere; nosZ gene; constructed wetlands

DISCUSSION

The understanding of the mechanisms leading to the effective removal of contaminants is of particular importance in treatment wetlands designed for final polishing purposes. The situation may be critical if nitrogen, and more specifically ammonium is the main compound to be removed from water to fulfil the quality standards expectations. The main reason for this is that an alternation of almost divergent environmental conditions is usually needed to promote nitrification and denitrification that will lead to a complete removal of nitrogen. Theoretically, free water surface wetlands provided with vegetated areas can supply such a range of conditions and may allocate the coupled process in distinct compartments. Additionally, plant roots may gather such spatial heterogeneity in smaller scales avoiding the need for bigger wetland areas with larger number of ponds.

In this work we have evaluated the composition and function of microbial communities of denitrifiers and ammonia oxidizing bacteria (AOB) associated with the presence of vegetation and the seasonal hydrodynamics of the wetlands. As shown in the previous section, the nutrient

dynamics of the wetland are strongly influenced by variables such as water loads that hamper the interpretation of molecular and activity data in terms of a single environmental factor. Nevertheless, plant driven mechanisms have been shown to be very important and explained most of the differences seen in the microbial diversity patterns and metabolic rates that occurred both between bare sediments and rhizospheres.

The *in vitro* obtained potential nitrification and denitrification rates provided useful values for sample comparison between rhizospheres of different plant species but can be blurry reflections of actual values found *in situ*. Potential activities measured in laboratory conditions in terms of dry weight were used to estimate nitrification and denitrification rates per square meter of wetland surface. For rhizosphere samples, calculations were done assuming a constant potential activity of the associated bacteria in all the surface area of living roots and rhizome regardless of depth, and using literature values for root biomass. Common figures of below ground biomasses vary from 0.4 to 0.7 Kg/m^2 for *Typha latipholia* and from 1.5 to 2.4 Kg/m^2 for *Phragmites australis* at similar wetland conditions than those observed in Empuriabrava CW (4, 99). For sediments without vegetation, estimates of potential activities per unit area were calculated from measured ratios of dry weight per unit surface considering the upper 4 cm of sediment. This area is thought to concentrate the majority of the microbial activity that takes place in the sediment, especially when looking at nitrification and denitrification (107). Estimated potential denitrification rates varied from 4.9 to 5.2 mg N/m^2/day in non vegetated sediments and added 0.9 mg N/m^2/day and 4.9 mg N/m^2/day in *Typha* sp. and *Phragmites australis* planted areas. In the case of potential nitrification, maximum estimated rates were 1.7 mg N/m^2/day, 0.05 mg N/m^2/day and 0.2 mg N/m^2/day for bare sediments and *Typha* sp. or *Phragmites australis* stands, respectively.

Estimated nitrification and denitrification rates at the optimal conditions in roots and rhizomes, revealed that plants had a lower impact than that predicted for laboratory experiments. This was particularly evident for nitrite+nitrate reduction rates. Whereas rates in sediment areas planted with *Typha* sp. may gain up to a 18% of potential activity, *Phragmites australis* would represent a much higher contribution to the activity in sediments, from a 80 up to a 90% increase. The effects on nitrification, on the other hand, would be less significant and would only account for increases of 1-4% for *Typha* sp. and 9-12% for *Phragmites australis* with respect to bare sediments, at the best conditions.

Results show evidences to consider planted areas, especially those dominated by *Phragmites australis*, as a fundamental factor for the efficient performance of the constructed wetland for the final denitrification step. The enhanced microbial activity relies in the production of root exudates, and the presence of a higher amount of organic matter due to plant litter, which would contribute to the increase of profitable carbon sources for heterotrophic bacteria, promoting denitrification in the absence of oxygen. Regarding nitrification, plant mediated stimulation of metabolic activity in the root surroundings is probably attributable to the release of oxygen, which would enable the development of ammonia oxidizing bacteria deeper in the sediment.

The higher capacity of plant roots to promote nitrogen removal was demonstrated in laboratory scale microcosms using samples obtained during spring and summer of years 2005 and 2006. It is interesting to note that potential activities of both nitrification and denitrification were preserved, and even enhanced, in periods of complete drainage and exposure of the wetland sediments to prolonged drying periods. As an example, during August 2006 a botulism outbreak affecting bird wildlife forced the drainage of the treatment cells. The drying period persisted for the whole month and left sediment surfaces and vegetation stands at intense drying conditions and temperatures well above 33ºC during daytime. The sediment and rhizosphere potential rates analysed under these circumstances indicated a clear response in *Typha* sp. rhizosphere. Nitrite+nitrate reduction and ammonia oxidation rates increased a four fold when compared with activity values obtained before and after the drying period. Conversely, potential rates measured at *Phragmites australis* rhizospheres remained at similar levels (Figure 1). This fact provides further evidence of the singular properties of a given plant species to support the metabolism of the underlying bacterial communities. Unfortunately, due to the lack of molecular analyses for samples retrieved in August, it remains unclear whether drying affected the community structure of denitrifiers and AOB. Nevertheless, flood interruptions do not seem to pose a major issue, since, when the system was returned to ordinary operation conditions in September 2006, the potential activity was restored satisfactorily, showing that CWs are a well buffered system and can tolerate drastic environmental perturbations.

The communities of denitrifying and ammonia oxidizing bacteria expressed characteristic dynamics to various external factors, and the conclusions that can be drawn for each population vary accordingly. With respect to AOB, a constant community pattern was generally found in all

retrieved samples from either bare sediment surface or reed and cattail rhizospheres, showing little influence of variables such as nutrient composition and load. Neither significant change in species composition was observed when plants were considered, despite the explicit effects in their metabolic capabilities. The structure of the community was reduced to a few genotypes, all of them belonging to *Nitrosomonas* clusters 6a and 6b (94). Closest cultured relatives according to partial sequences of the 16S rRNA gene were *Nitrosomonas marina*, *Nitrosomonas ureae* and *Nitrosomonas oligotropha*. With the methods used, it was evident that changes in the potential nitrification in roots are not due to a different species composition but to changes in either the specific activity of cells or increased population densities at the root surface. However, many works report the use of other molecular markers that should be used to confirm this data. An option is to test a functional marker such as the *amoA* gene (A subunit of the ammonia monooxigenase). Comparisons done with *amoA* and CTO primer pair amplified 16S rDNA genes, have shown that a larger number of AOB lineages were found by the *amoA* primers, indicating that a higher diversity was revealed by this primer pair. Also, the use of *amoA* restricted the amplification of inespecific sequences (84).

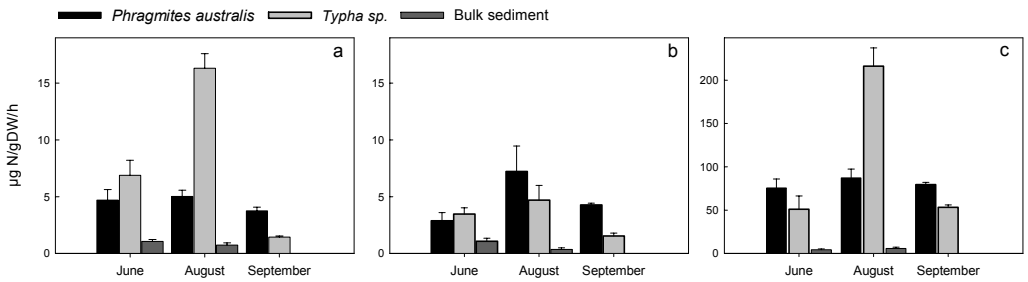


Figure 1. Seasonal variations in the activity for nitrogen removal in samples of sediment and rhizospheres of *Phragmites australis* and *Typha* sp. of the CW measured in laboratory incubations. Rates for: a) NO_2 production, b) NO_3 production, and c) denitrification (NO_3+NO_2 reduction) are given in µg N/g DW.h and expressed as mean values ± standard error.

The molecular analysis of denitrifying bacteria is limited to the use of functional genetic markers due to the wide distribution of this physiological trait among many phylogenetic lineages. All distinct molecular approaches employed in this work coincided and revealed significant rearrangements of the community structure of denitrifying bacteria as a response to environmental modifications, caused either by a vegetation effect or environmental conditions. The estimated Shannon-Weaver diversity index of *nir*S type containing denitrifier community obtained in the

bare sediments for the period 1998-2000 increased in situations of relatively short water retention times and high nutrient loads. It is worth to note that these samples belonged to the onset of the wetlands and many changes in environmental parameters occurred which can not be completely overruled as factors controlling the diversity of denitrifying bacteria. Increases in diversity were likely to occur in periods of high nutrient loads since they coincided with the enrichment of the ammonia content in the influent, which would have favoured a higher diversification of denitrifiers due to the lack of nitrate as readily utilizable substrate. In comparison, at conditions where a nitrate enriched influent was applied to the wetlands, a few and better adapted populations were supposed to be selected.

The use of both T-RFLP and DGGE as experimental approaches has led to the same conclusions. Although it can be deduced that plants indeed play a significant role, sampling time seemed to prevail as the driving force defining denitrifying populations in the sediments. As stated in chapter 3.2 the Empuriabrava FWS-CW suffered from many operation adjustments leading to drastic changes in the water flow, retention times and nutrient loads. The marked seasonal trend in the hydraulic regime affects the system as a whole, and thus it is reasonable to assume that the denitrifiers, either in sediments or rhizospheres, would express a global response depending on the prevalent environmental conditions. It should be considered that the observed seasonal patterns may also involve other biological mechanisms related with plant physiology and growth cycle. A dependence of denitrifiers on the plant species was also suggested when minor populations were considered. Consistently, the differences between *Typha* sp. and *Phragmites australis* rhizospheres tended to be more pronounced during occasions of high nutrient discharge, showing a reflex of varying potential denitrification rates. It is not clear to what extent plants will affect activity and composition of microorganisms thriving in the rhizosphere as a result of variations in the nutrient composition of the overlying water, and many of the observed changes may respond to nutrient translocation at the root surface (119). Root exudates vary temporally and spatially along the root (55, 64), and the effects of the release of easily degradable sugars and organic acids during the growth of new roots would have probably had a more significant impact on denitrifying communities in June compared to September 2006.

Although the use of genetic diversity studies opened a countless number of opportunities to go forward in the study of microbial community diversity and dynamics, one relevant aspect that

should not be forgotten in microbiology is the outstanding need to obtain pure cultures of new isolated microorganisms. The challenge is to develop new and improved protocols for the current culture and isolation techniques to overcome the existing bias of non-culturing based approaches and capture a wider portion of the bacterial diversity. One of the unsolved questions regarding the diversity of denitrifying bacteria in the sediments of the Empuriabrava CW, is the existence of Cu-containing nitrite reductases (NirK) harbouring bacteria. As derived from molecular analyses the presence of these microorganisms is meant to be quite low, although its presence can not be completely ruled out from negative PCR results. An additional attempt to analyze the presence of *nir*K-type nitrite reductase containing microorganisms was done by culturing techniques.

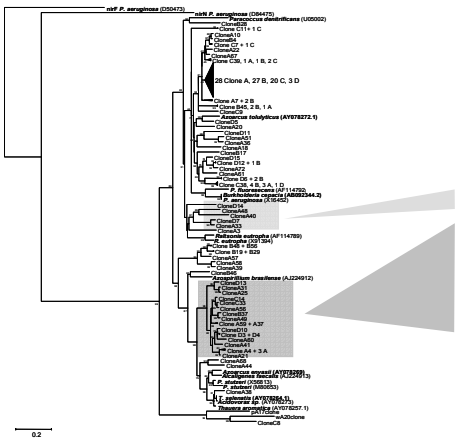
The test of mineral and organic formulated media allowed us to successfully enrich and isolate 22 bacterial strains indigenous to the sediments and rhizospheres of the Empuriabrava CW (Table 1). The PCR detection of *nir*S or *nir*K genes confirmed that the 68% of isolates were indeed true denitrifiers possessing nitrite reductase genes, and at least three of them were *nir*K-type denitrifiers, which we formerly had not been able to detect in sediment samples with the primer combinations used. The partial sequencing of 16S rRNA gene of *nir*K-containing isolates yielded high homologies (>98%) with sequences obtained from the plant related bacterium *Agrobacterium tumefaciens* strain UP-3 (AY364329), the salt marsh rhizosphere isolated *Erwinia* sp. MK01 (AY690711) and *Aeromonas media* (AF418217). Although it has not been confirmed experimentally for the isolated strains BN-2 and BN-8, *Erwinia* sp. and *Aeromonas media* have not been described as nitrite reducing bacteria and the presence of non-functional *nir*K genes or unspecific PCR products has to be elucidated.

It remains unclear whether *nir*S or *nir*K-containing denitrifiers vary in abundance and distribution over the sediment surface and rhizhospheres, but the observed results indicate the strong need to test several primer combinations and PCR amplifying conditions to ascertain the true presence and diversity of a particular group of bacteria.

Concerning similarities of functional genes, *nir*S and *nir*K sequences of isolated strains showed low percentage similarities (below 82%) with previously published sequences from cultured bacteria, proving the effectiveness of the method in isolating new denitrifiers and at the same time reflecting the yet hidden diversity of denitrifiers in the sediment. The evaluation of the *nir*S sequences presented high homologies to those retrieved from the cloning analysis presented in

chapter 1 and grouped accordingly in the phylogenetic tree, primarily in clusters Ic, IIb and IIc. However, the majority of newly isolated representatives showed high dissimilarities with the *nir*S sequences obtained from cloning of sediment samples, giving further proof of the bias between culturing and genetic approaches used to study the natural diversity. The potential activity of these strains remains yet to be tested, but the characterization of isolated microorganisms is a fundamental aspect since it will permit a better understanding of their physiology and reveal how identity and function are related.

Table 1. Cultures of bacteria isolated from the CW using mineral (M) or organic (O) media formulation. Identification according to the results of a BLAST search (~800 bp fragment of 16S rRNA gene) is shown. The presence of either *nirS* or *nirK* genes was confirmed by PCR. *nirS* genes were sequenced (~890 bp fragment) and compared with previously retrieved sequences from sediments of the CW (see arrow heads showing the connection to clusters within the phylogenetic tree). Homologies found ranged from 85 to 98 % on a nucleotide-nucleotide basis comparison.



Media	Isolate	Maximum similarity gb accession number—(similarity ~ 800 bp fragment)	*nirS*	*nirK*
M	N-13	AF011346 *Azovibrio restrictus* (99 %)	√	
M	N-3	AY315159 Glacier bacterium FJS32 (97 %)	√	
M	N-3G	AY537451 Uncultured bacterium clone mek63f09 (96 %)	√	
M	N-4	AY212681 Uncultured bacterium clone 234ds5 (98 %)	√	
M	N-7	AY689069 *Aeromonas* sp. 9C_12 (99 %)	√	
M	N-12	— not determined —	√	
O	BN-12	— not determined —	√	
O	BN-13	AB184266 Streptomyces cinnabarinus (99 %)	√	
M	N-1	AF423281 Uncultured soil bacterium clone (97 %)		
M	N-1G	AY928226 Bacterium BH98-203 (99 %)	√	
M	N-2C	AJ864847 *Acidovorax* sp. J33 (100 %)		
M	N-5	AY928473 *Aeromonas veronii* bv. *veronii* (99 %)		
M	N-10	AY364329 *Agrobacterium tumefaciens* strain UP-3 (98 %)		√
M	N-11	AY064177 Uncultured beta proteobacterium (99 %)	√	
M	N-13.2	DQ115791 *Rahnella* sp. HX2 (99 %)	√	
O	BN-4	AF463533 *Citrobacter* sp. TSA-1 (99 %)	√	
O	BN-5	AY567708 Candidatus *Cuticobacterium kirbyi* (99 %)		
O	BN-14	DQ166817 Aeromonas hydrophila strain FN100 (99 %)		
O	BN-1	AB078067 *Flexibacter sancti* (98 %)		
O	BN-2	AF418217 *Aeromonas media* (99 %)		√
O	BN-8	AY690711 *Erwinia* sp. MK01---99%		√
O	BN-9	AY376702 Uncultured bacterium clone O3 (99 %)		

The studies presented provide some valuable background to state that indeed plants may well intervene in the nitrogen removal in constructed wetlands, not only by facilitating general retention mechanisms but also by direct influence on the activity and also community structure of the microorganisms implicated in the biochemical transformations. Particularly, we have shown

that plant rhizospheres impact potential bacterial nitrification and denitrification activities in laboratory incubations using fully homogenized sediment-water slurries. At optimal, substrate saturating conditions potential nitrification occurred at a significantly lower speed than nitrate removal, indicating that this activity may be a limiting process in the overall nitrogen removal at high ammonium loads. Also under these circumstances, nitrification and nitrate removal rates measured at *Typha* sp. rhizospheres were significantly lower than those found for *Phragmites australis*. The plant coverage effect on three parallel cells of a FWS-CW treatment wetland has been evaluated from chemical measurements of nitrate, nitrite and ammonia at the inlet and outlet water. Our findings suggest that a wise use of plant coverage, involving placement, density and plant species composition may be a valuable tool to attempt an upgrade of the treatment performance at the Empuriabrava CW and increase the water re-use yields. An increase of plant density of common reed and cattail at the inlet of treatment cells coincided with best contaminant removal rates. The results obtained in vitro suggest that an improvement of nitrification and denitrification may be accomplished by increasing the proportion of *Phragmites australis*, especially at the inlet area of the cell. Denitrification activity has not been identified as a key factor for *in situ* nitrogen removal providing that this process was generally well buffered despite changing environmental conditions.

However, our results derive mainly from laboratory incubations, which may not necessary reflect the actual situation at the treatment cells. The next step in the research in the Empuriabrava CW will attempt to move deeper into microbial function and will involve *in situ* measurements of potential activities to better correlate with real functioning of the treatment system. The main aspects that are currently being developed or will be in the near future in the Empuriabrava project include:

- The estimation of *in situ* nitrate+nitrite reduction and nitrification activities using closed mesocosm chambers in the sediment.
- The test of markers based on functional genes such as the *amoA*, *amoB* or *amoC* genes, to attempt to retrieve a higher diversity of ammonia oxidizing Bacteria and check for differences related to plant species or seasonal variations.

- Include the analysis of communities of ammonia oxidizing *Archaea*, which have been recently found to numerically dominate in marine and soil ecosystems
- The evaluation of gene expression by the analysis and quantification of *nosZ* and bacterial and archaeal *amoA* transcripts in the sediment and rhizosphere under conditions of enhanced activities.

5 CONCLUSIONS

1) The analysis of partial *nirS* gene sequences revealed a high diversity of denitrifiers in the sediments of the CW. Similarity values indicated a high number of sequences distantly related to cultured reference organisms. This fact stresses the need for enhanced culturing techniques to characterize physiologically the community of denitrifiers.

2) Environmental heterogeneity had a major impact in the establishment of characteristic denitrifying populations in sediments and rhizospheres within the CW. A plant specific effect was proven as a factor determining the composition of denitrifying bacterial communities in the rhizosphere compartment when *nos*Z was used as a molecular marker.

3) Ammonia Oxidizing Bacteria community exhibited a low complexity in bulk sediment and rhizosphere samples, and no significant shifts were detected at either time or space scales.

4) With the methods used in these studies, no link could be detected between changes in the community structure and potential activities measured at the sediment. All methods without exceptions are subjected to limitations, and a greater insight in the analysis of functional bacterial groups related to nitrogen removal is still needed to fully understand these relationships.

5) Potential nitrogen removal rates were considerably higher in the rhizospheres compared to bulk sediment samples, when expressed per gram of dry weight of roots and sediment, respectively. Estimated potential activities in terms of sediment surface area of vegetated and non vegetated zones confirmed the significant contribution of *Phragmites australis* stands to increased nitrification and denitrification rates.

6) The transient accumulation rate of nitrite in non vegetated sediments and *Typha* sp. rhizosphere suggests a low prevalence of nitrite oxidizing bacteria (NOB) in these microenvironments.

7) Potential activities within a sample type stayed over time for the episodes analyzed, suggesting the wetlands are able to tolerate drastic changes in the operational conditions.

8) Plant specific changes in potential nitrification and denitrification activities between *Typha* sp. and *Phragmites australis* rhizospheres were only measured when stress (i.e. high nitrogen loads or extended drying periods) situations were considered.

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