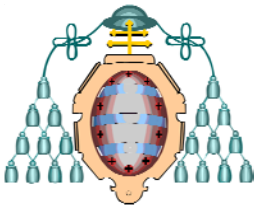
UNIVERSIDAD DE OVIEDO
Departamento de Biología de Organismos y Sistemas

Caracterización de la materia orgánica en suelos y sedimentos de ambientes estuarinos

Memoria de Tesis Doctoral

Cristina Santín Nuño

2008



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Ordenación del Territorio
UNIVERSIDAD DE OVIEDO

Tesis Doctoral

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Capítulo III: Santín, C., González-Pérez, M., Otero, X.L., Vidal-Torrado, P., Macías, F., Álvarez, M.A., 2008. Characterization of humic substances in salt marsh soils under sea rush (*Juncus maritimus*). Estuarine, Coastal and Shelf Science 79, 541-548.

Capítulo IV: Santín, C., González-Pérez, M., Otero, X.L., Álvarez, M.A., Macías, F. Humic substances in salt marsh soils colonized by *Spartina maritima*. Estuarine, Coastal and Shelf Science. In press.

Capítulo V: Santín C., de la Rosa, J. M., Knicker, H., Otero, X. L., Macías, F., Álvarez, M. Á., González Vila, F.J. Effects of reclamation and regeneration processes on organic matter from estuarine soils and sediments. Enviado a Organic Geochemistry.

Capítulo VI: Santín, C., Yamashita, Y., Jaffé, R., Otero X.L., Macías, F., Álvarez, M.A. Characterizing humic substances from estuarine soils and sediments by excitation-emission matrix spectroscopy and parallel factor analysis. Enviado a Biogeochemistry.

Capítulo I. Introducción General

1.- Los humedales costeros

Una de las definiciones más ampliamente aceptada de humedal comprende *"todas aquellas extensiones de marismas, pantanos y turberas, o superficies cubiertas de agua, sean éstas de régimen natural o artificial, permanentes o temporales, estancadas o corrientes, dulces, salobres o saladas, incluidas las extensiones de agua marina cuya profundidad en marea baja no exceda de seis metros"*. (Artículo 1.1 de la Convención Ramsar).

Los humedales representan uno de los ecosistemas más valiosos y a la vez frágiles de nuestro planeta y, a pesar de que cubren tan solo un 1.5 % de la superficie terrestre, se ha estimado que contribuyen al 40% de los servicios ecosistémicos renovables a nivel planetario (Costanza et al., 1997). Entre las numerosas funciones ecológicas y ambientales que cumplen estos sistemas pueden destacarse las siguientes (Schnack, 2001):

- Elevada productividad
- Sumidero de carbono atmosférico
- Filtro y retención de elementos contaminantes
- Retención y exportación de sedimentos y nutrientes
- Control de la erosión
- Reducción de los máximos de avenidas e inundaciones
- Estabilización de costas y microclimas
- Depuración de aguas y recarga de acuíferos
- Refugio de vida silvestre y reservorio de biodiversidad
- Sostenimiento de pesca, ganadería y agricultura
- Educación, recreación, y valor cultural

El presente estudio se centra en los humedales costeros (aquellos localizados en una cuenca de drenaje costera) y, más concretamente, en zonas de marismas de estuarios y rías de la costa cántabro-atlántica española.

Las características y distribuciones de los humedales costeros a lo largo del litoral español dependen, entre otros factores, de la dinámica marina, la evolución geológica y el clima. Estos factores son los

principales responsables de las diferencias que presentan los humedales costeros cántabro-atlánticos, esencialmente rías y estuarios, con los mediterráneos, donde los más representativos son los deltas, las lagunas costeras y las albuferas.

La marcada diferencia en el rango mareal entre la costa cántabro-atlántica y la mediterránea explica en gran medida la predominancia de distintos tipos de humedales en los dos litorales de la Península Ibérica. Por un lado, el rango mareal de entre 2 y 4 m de altura que presenta el océano Atlántico invade una amplia franja del litoral y parte de las desembocaduras fluviales, generando sistemas parcialmente sumergidos por el mar como las rías y los estuarios y una gran gama de ambientes diferentes en las zonas intermareales. En estas desembocaduras, la actividad de transporte y redistribución de sedimentos aportados por los sistemas fluviales es elevada y a la vez muy variable, dependiendo de las características y extensión de la cuenca hidrográfica. Por otra parte, el rango mareal inferior a 2 m que caracteriza a la costa mediterránea hace que los agentes de transporte marinos no tengan tanta capacidad de redistribución, de forma que existe un predominio de la acumulación de material frente al de erosión marina, dando lugar, entre otros ambientes, a los deltas.

Las desembocaduras fluviales en la costa cantábrica (provincias de Lugo, Asturias, Cantabria, Vizcaya y Guipúzcoa) corresponden, en la mayoría de los casos, a estuarios barrera (Fairbridge, 1968). Estos estuarios se caracterizan por ser estrechos y alargados hacia el continente y estar semicerrados por un cierre rocoso, por un cuerpo sedimentario o por un sistema de playa y dunas. Hidrológicamente, están dominados por la componente fluvial, restringiéndose el predominio mareal a la zona más externa y próxima al mar. A lo largo del estuario existe un relleno fluviomareal sobre el que se desarrollan ambientes como llanuras intermareales y marismas.

Las rías son otras de las formaciones características en las desembocaduras fluviales de la costa cantabro-atlántica española, si bien su desarrollo se limita prácticamente a la costa atlántica gallega. Dentro del litoral gallego encontramos las Rías Bajas, que corresponden a zonas

invadidas por el mar debido al hundimiento de bloques litosféricos como consecuencia de esfuerzos tectónicos ocurridos después de la orogenia alpina y al posterior aumento del nivel del mar; y las Rías Altas, que corresponden a valles fluviales cuya desembocadura fue parcialmente invadida por el mar. En líneas generales, la influencia mareal en las rías es mayor que en los estuarios, limitándose el ámbito estuarino a su parte más interna (Evans y Prego, 2003).

Los sistemas estuarinos, transicionales entre el medio terrestre y el marino, poseen una elevada variabilidad y diversidad, al presentar rasgos de cada uno de los medios que en ellos convergen, además de características propias e irrepetibles. El grado de inundación mareal, la influencia fluvial y la desigual distribución del sedimento dentro del ámbito estuarino dan lugar a múltiples y muy diversos ambientes, entre ellos las marismas.

2.- Las marismas

Puede entenderse por marismas aquellos humedales dominados por plantas vasculares emergentes y donde la vegetación es principalmente herbácea (Zedler, 2000). Más concretamente, en referencia a las marismas costeras, existe sobre ellas influencia del agua del mar y por tanto, su vegetación presenta, en mayor o menor medida, tolerancia a la salinidad. Además, algunos autores precisan que las marismas costeras se extienden desde el nivel medio de pleamares muertas hasta el nivel máximo de pleamares astronómicas (Long y Mason, 1983).

2.1. Formación y clasificación de las marismas

La figura 2 muestra de forma esquemática las tres fases fundamentales del proceso de formación y evolución de una marisma según Long y Mason (1983).

Llanura intermareal (Figura 2. A)

Se localiza en la zona intermareal más baja y es inundada con cada marea. El prolongado periodo de inundación al que están sometidas

estas zonas condiciona el asentamiento de la vegetación, representada preferentemente por algas, aunque también pueden encontrarse plantas vasculares como *Zostera noltii*, ocupando biotopos que quedan descubiertos en la bajamar (Fig. 3.A), y *Zostera marina*, permanentemente sumergida.

Marisma baja o pionera (Figura 2. B)

Cuando la altura de las llanuras intermareales alcanza el nivel medio de las pleamares muertas, permitiendo la colonización por vegetación vascular, empieza a desarrollarse la marisma baja. Las plantas pioneras favorecen la sedimentación mediante la retención de sedimento, disminución de la erosión y aporte de materia orgánica. De esta forma, la colonización vegetal contribuye a la evolución y formación de suelos más estructurados.

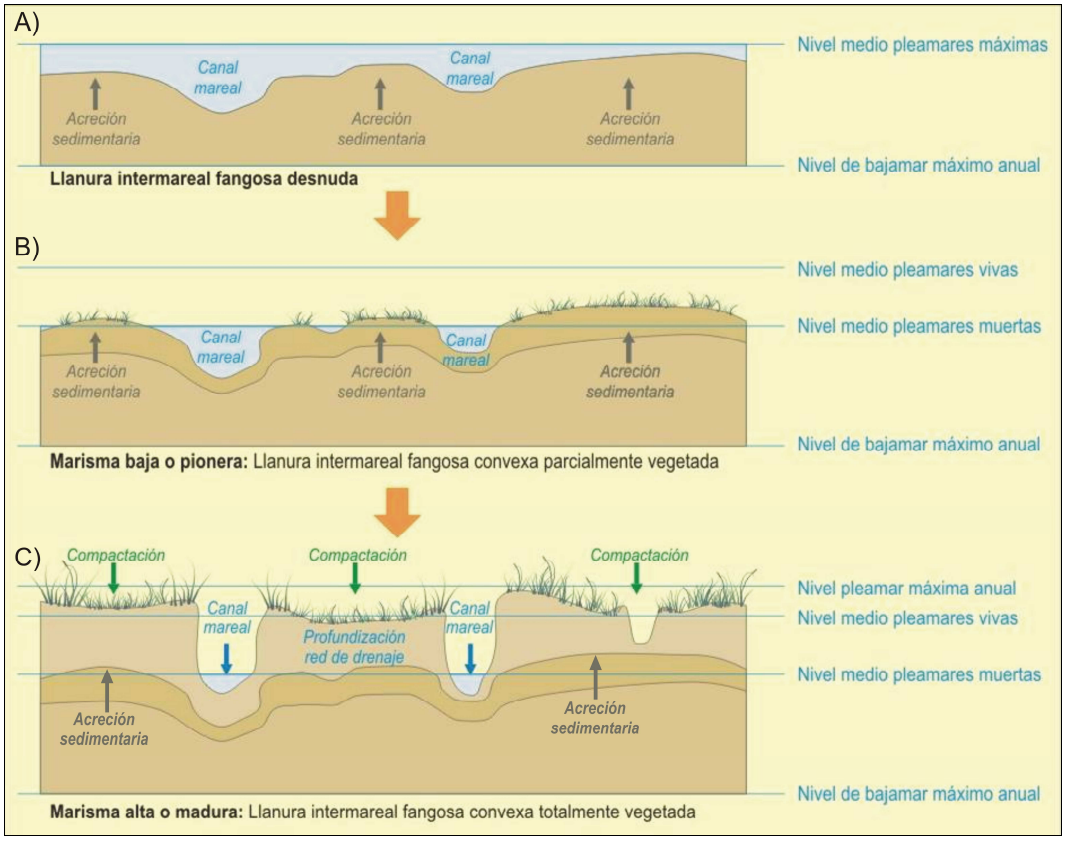

Figura 2. Formación y evolución de las marismas (modificado de Long y Manson, 1983).

La marisma baja ocupa la zona intermareal media, permaneciendo inundada durante prácticamente toda la pleamar, ya que es la primera zona en ser inundada durante la subida de la marea y la última en quedar

expuesta en el descenso de la misma. Esta elevada actividad mareal genera sustratos inestables y modelos de drenaje y micro-relieve cambiantes a corto plazo, lo cual limita el desarrollo de la cubierta vegetal.

Como especie representativa se puede destacar a la hierba salada (*Spartina maritima*) (Fig. 3. B). Esta gramínea perenne, excelente pionera y fijadora de sedimento, forma manchas monoespecíficas y coloniza suelos con un alto grado de inundación y condiciones fuertemente reductoras. Aunque predominante en la marisma baja, también puede encontrarse en pequeñas depresiones con suelos encharcados de la marisma alta (Otero, 2000).

Figura 3. A) Llanura intermareal colonizada por *Zostera noltii* en el Estuario de Villaviciosa.

Figura 3. B) Mancha monoespecífica de *Spartina maritima* en una marisma baja de la Ría de Ortigueira.

Marisma alta o madura (Figura 2. C)

Conforme progresa la acreción vertical, los suelos de la marisma se compactan y desarrollan y la influencia mareal se reduce, lo que permite el asentamiento de nuevas especies vegetales. Al mismo tiempo, la red de drenaje de canales mareales se va encajando y la vegetación contribuye a fijar los márgenes de los canales.

La marisma alta o madura se sitúa en el sector más elevado de la zona intermareal, siendo inundada menos frecuentemente y con canales mareales menos ramificados que los de la marisma inferior. Las condiciones de salinidad y redox son muy variables tanto espacial como temporalmente, dependiendo de la influencia de la marea y del aporte de agua dulce, así como de los cambios estacionales de la

evapotranspiración (Otero, 2000). Este aspecto da origen a una elevada variabilidad de ambientes. Por ello, es en la marisma alta donde encontramos mayor diversidad de comunidades y especies vegetales. Además, corresponden a las zonas del estuario más modificadas y utilizadas para usos antrópicos.

Brevemente describiremos dos especies vegetales, ampliamente presentes en la marisma alta, que serán sujeto de estudio en posteriores capítulos.

Halimione portulacoides: este arbusto halófilo encuentra su hábitat óptimo en suelos bien aireados, preferentemente zonas con depósitos arenosos o con un denso sistema de canales que facilite el drenaje (Benito, 1991). Presenta un porte de hasta 1.5 m de altura y hojas suculentas con un característico color verde grisáceo (Fig. 4. A). Se encuentra principalmente formando parte de la asociación *Halimionetum portulacoidis* (Bueno, 1996).

Figura 4. A) Marisma alta vegetada con *Halimione portulacoides* en el Estuario de Urdaibai.

Figura 4. B) Juncal marítimo en la marisma alta del Estuario de Foz.

Juncus maritimus: su óptimo de desarrollo se da en la marisma alta aunque también puede aparecer en zonas de transición con la marisma baja (Sánchez et al., 1998). El junco marítimo es una planta perenne, de hasta 1 m de altura, con tallos y hojas cilíndricos y un poderoso rizoma horizontal. El grado de salinidad que tolera es muy amplio y dependiendo

de la posición que ocupe en el estuario se puede encontrar junto a diferentes especies acompañantes, formando distintas asociaciones vegetales (Ej.: *Junco maritimi-Caricetum extensae, Agrostio stoloniferae-Juncetum maritimae, Puccinellio maritimae-Juncetum maritimi*) (Bueno, 1996) (Fig. 4. B).

2.2. Suelos vs. Sedimentos

La *Soil Taxonomy* (Soil Survey Staff, 1999) define como suelo aquella entidad natural presente en la superficie terrestre que ocupa un espacio y está compuesta por una fase sólida (minerales y materia orgánica), otra líquida y una tercera gaseosa. Además, el suelo se caracteriza por cumplir, al menos, uno de los siguientes requisitos: i) estar dividido en horizontes diferenciados del material original como resultado de adiciones, pérdidas, transferencias y transformaciones de materia y energía; ii) presentar la capacidad de albergar plantas vasculares en condiciones naturales.

El límite superior de los suelos puede darse entre este y: la atmósfera; aguas someras; plantas vivas o materia orgánica muerta. Las áreas cubiertas permanentemente por aguas profundas (> 2.5 m) no se consideran suelo pese a poder existir asentamiento de plantas vasculares (Soil Survey Staff, 1999).

Cuando se estudian ambientes transicionales entre sistemas acuáticos y terrestres como las marismas existen diferentes acepciones de suelos y sedimentos según la bibliografía consultada (más detalles en Ferreira et al., 2007), ya que, atendiendo a la propia definición de suelo, las marismas corresponden al límite inferior de la distribución del suelo en la superficie terrestre.

En la presente memoria se definen como suelos todos aquellos sustratos cuyas características permiten el asentamiento de plantas vasculares (Ej. Figura 5. A); y sedimentos a los sustratos desnudos o vegetados por algas y que sufren periodos de inundación prolongados (Ej. Figura 5. B).

Figura 5. A) Testigo de suelo de la marisma alta.

Figura 5. B) Testigo de sedimento de un canal mareal.

2.3. El reclamado

Desde la antigüedad las sociedades humanas han ocupado las regiones costeras por ser zonas fértiles, de fácil acceso y cercanas al mar. Esto, a lo largo de la historia, ha determinado que estas zonas hayan sufrido un intenso impacto por la acción del hombre. Así por ejemplo, se estima que en los últimos dos siglos, los humedales costeros españoles han visto reducida su superficie a menos de la mitad (Casado y Montes, 1995).

Los elevados valores ambientales de los humedales costeros contrastan con su extremada fragilidad, ya que son sistemas particularmente sensibles a usos inadecuados o excesivos. En el litoral cantabro-atlántico, el progresivo deterioro de los humedales costeros se ha visto favorecido por el escaso control del desarrollo urbanístico y de la política de infraestructuras, por una insuficiente protección frente a la contaminación y a los vertidos incontrolados de residuos así como por la alteración de estos sistemas para adecuarlos a usos antrópicos (Indurot, 2003).

Dentro de los ambientes costeros, las marismas estuarinas son unos de los más afectados por la acción humana. Consideradas hasta

hace poco zonas insalubres y necesitadas de ser saneadas, han sido drásticamente mermadas mediante el proceso descrito como reclamado o polderización. En este proceso, la influencia del mar en el área de marisma se reduce o elimina mediante la construcción de un muro o dique; posteriormente, la zona es drenada y a veces se aplican enmiendas al suelo o se añaden materiales de relleno. Una vez el proceso de reclamado se completa, la zona está acondicionada para usos agrícolas o ganaderos, e incluso para fines urbanos. El área reclamada es designada como pólder, que proviene de la palabra holandesa *polder* (terreno ganado al mar), aunque existen también localismos como el de "porreo" en Asturias o el de "itzak" en el País Vasco.

En el año 2003, el Instituto de Recursos Naturales y Ordenación del Territorio de la Universidad de Oviedo (Indurot) abordó, por encargo de la Dirección General de Costas (Ministerio de Medio Ambiente, Gobierno de España), el estudio y evaluación de 85 desembocaduras fluviales a lo largo de la costa cantábrica. En este estudio se puso de manifiesto que un 38% de los sistemas dunares y un 48% de los ambientes estuarinos han sido eliminados por rellenos y edificaciones, lo que suma un total de 8500 ha de hábitats de interés comunitario destruidas. Además, la mitad de los ambientes estuarinos que aun se conservan se encuentran alterados, en mayor o menor grado, por la presencia de barreras mareales y procesos de reclamado.

Cuando las tierras estuarinas son reclamadas las condiciones edáficas cambian drásticamente. Con la alteración del régimen de inundación y el drenaje del terreno se establece un ambiente aeróbico en el que puede producirse la oxidación de los sulfuros de hierro, generalmente pirita. Esta oxidación trae como consecuencia una fuerte disminución del pH del suelo, pudiendo llegar a generar suelos sulfato-ácidos (Van Breemen, 1982). La acidificación de los suelos conlleva además la solubilización de Fe, Mn, Al, y metales traza. El aumento de la biodisponibilidad de estos elementos puede suponer un problema de toxicidad en algunos ambientes (Otero y Macías, 2003).

Otra consecuencia del desecado de los suelos es que, en un ambiente oxidante, la mineralización del carbono es hasta tres veces más

rápida que en ambientes pobres en oxígeno (D'Angelo y Reddy, 1999). En esta situación, la tasa de descomposición de la materia orgánica puede superar a la de producción, lo que provocaría una disminución de la materia orgánica retenida. Sin embargo, como veremos en posteriores capítulos, los ambientes estuarinos son extraordinariamente variables a una escala espacial muy reducida. Por tanto, la dirección que tomen los cambios después del proceso de reclamado está condicionada por multitud de factores, no pudiéndose llegar a predecir de un modo general.

3.- El sujeto de estudio: la materia orgánica edáfica

3.1. Definición y funciones

La materia orgánica edáfica (MOS) es sujeto de muchas y diversas definiciones, aunque no siempre con la precisión adecuada. Así, por ejemplo, la FAO (2005) considera MOS "*cualquier material producido originalmente por un organismo vivo (planta o animal) que vuelve al suelo y pasa a través del proceso de descomposición"*. La principal limitación de esta definición es la de no considerar una parte fundamental de la materia orgánica en los suelos: los microorganismos, tanto vivos como muertos, así como los compuestos derivados de ellos.

Una definición más apropiada y más amplia a la vez, sería la propuesta por Coleman et al, (1989): "*la materia orgánica edáfica es una mezcla de componentes de diferentes formas y con diferentes tipos de orígenes bióticos"*; ó la de Stevenson (1994): *"la materia orgánica edáfica comprende el conjunto de materiales orgánicos presente en los suelos"*.

La materia orgánica es un elemento esencial de los suelos y un claro indicador de la calidad de los mismos. Su papel en la fertilidad del suelo es clave, así como en la retención de agua, cohesión de la estructura edáfica, resistencia a la erosión, retención de elementos contaminantes, sistema tampón o actividad biológica (Stevenson, 1994; Zech et al., 1997). Además, su principal componente es el carbono orgánico, por el cual el suelo constituye uno de los reservorios de carbono más importantes a escala planetaria (Lal, 2001).

3.2. Componentes de la materia orgánica edáfica

En líneas generales, la MOS puede dividirse**,** en primer lugar**,** en materia orgánica muerta y organismos vivos (Figura 6). El componente vivo de la MOS puede llegar a representar el 4% del carbono orgánico edáfico e incluye tanto macro como microorganismos. Por otra parte, la MOS muerta puede ser subdividida a su vez en materiales poco alterados y productos transformados. Los materiales poco alterados son principalmente residuos vegetales en diferentes fases de descomposición y en los que todavía se puede reconocer parte de su estructura original. Los productos transformados comprenden las sustancias no-húmicas (compuestos orgánicos bien definidos y caracterizados, como los carbohidratos, los lípidos o las proteínas) y las sustancias húmicas (SH). Estas sustancias húmicas se dividen fundamentalmente según su solubilidad en: ácidos fúlvicos (solubles en todo el rango de pH), ácidos húmicos (solubles a pH básico) y humina (insoluble) (International Humic Substances Society, 2007).

En los últimos años el concepto de “sustancias húmicas” ha sido ampliamente revisado (Sutton y Sposito, 2005) y, el más aceptado actualmente es el del “modelo supramolecular” propuesto por Piccolo (2001). Este autor define las sustancias húmicas como asociaciones de moléculas de pequeños tamaños estabilizadas por interacciones hidrofóbicas, puentes de H y fuerzas de van der Waals y derivadas de la degradación y descomposición de materia orgánica muerta.

Hay que tener en cuenta que la división de las SH en tres tipos (ácidos fúlvicos, húmicos y humina) es meramente operacional y no implica la existencia de tres tipos distintos de moléculas orgánicas (Hayes et al., 1989). Sin embargo, esta clasificación sigue siendo muy utilizada ya que, debido a la extrema complejidad de la materia orgánica, la extracción de una parte de esta, según criterios estándar, permite una separación coherente y homogénea, facilitando su estudio.

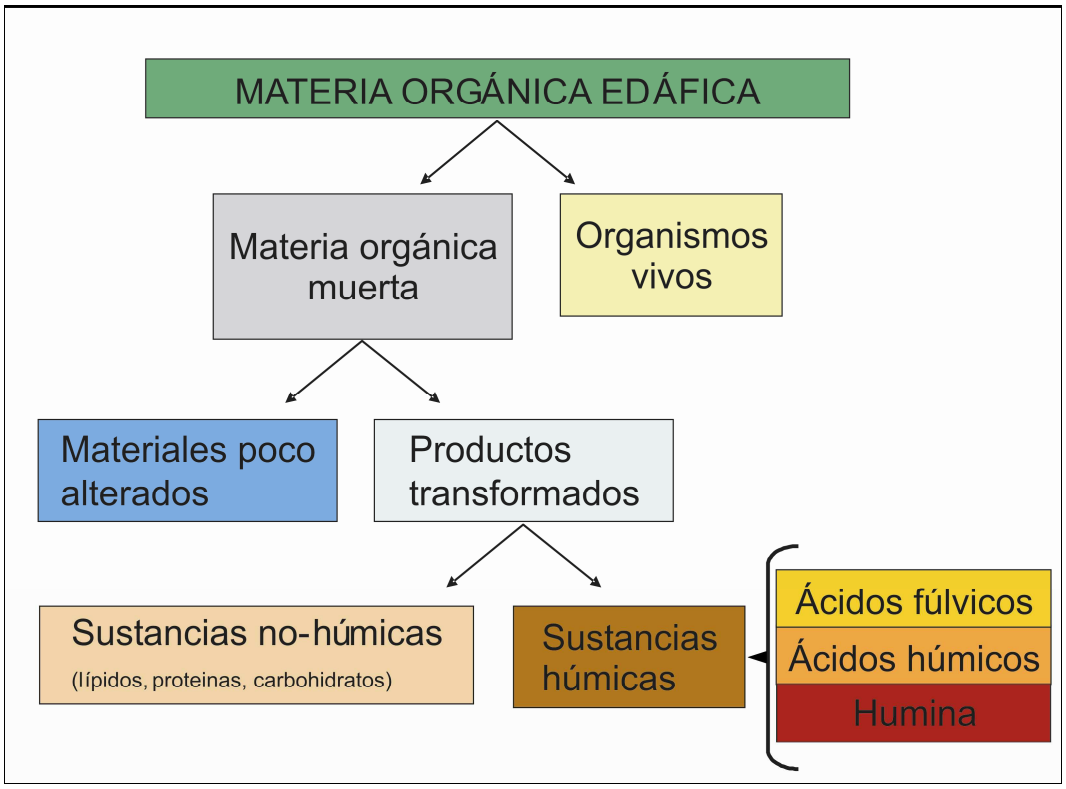

Figura 6. Componentes fundamentales de la materia orgánica edáfica (modificado de FAO, 2005).

3.3. Transformación de la materia orgánica edáfica

Una vez en el suelo, el proceso de descomposición de los materiales orgánicos es clave para permitir la continuidad de los ciclos biogeoquímicos del C y de otros elementos asociados (N, P, S,...). La descomposición de la materia orgánica es altamente compleja, presenta diferentes fases, y engloba, a su vez, varios procesos: humificación, mineralización, estabilización e inmovilización:

- *Mineralización*: transformación de compuestos orgánicos en compuestos minerales sencillos (Ej.: CO_2, H_2O, CH_4, NH_4^+, NO_3^-, SO_4^{2-}, HPO_4^{2-}). Es el fin último de todo elemento orgánico.
- *Humificación*: estabilización prolongada de los compuestos orgánicos frente a la degradación, mediante procesos tanto bióticos como abióticos.
- *Inmovilización*: proceso por el cual los microorganismos utilizan nutrientes del suelo para cubrir sus necesidades, fijándolos en su biomasa.

La cantidad y características de la MOS dependerán, por tanto, de los procesos previamente descritos, de los aportes de materiales orgánicos y su composición, así como de un gran abanico de factores ambientales físicos, químicos y biológicos.

3.4. Materia orgánica en marismas

Las marismas están entre los ecosistemas más productivos de nuestro planeta (Whitaker, 1975; Goñi et al., 2003), con una productividad primaria, en muchos casos, que supera a la de los bosques, los pastizales o las tierras de cultivo (Schlesinger, 1977). Por otra parte, en estos humedales existen frecuentemente suelos encharcados periódicamente; esto propicia un ambiente pobre en oxígeno en el que el proceso de descomposición de la materia es lento, permitiendo así que la tasa de producción supere con creces a la de descomposición (Troeh et al., 1999). El resultado es una acumulación neta de materia orgánica en suelos y sedimentos que puede llegar en algunos casos a varios metros de espesor. Por tanto, podemos referirnos a los suelos de marismas como trampas o sumideros de carbono (Chmura et al., 2003).

La estabilidad del carbono en los suelos está estrechamente ligada a las características de la MOS de la que forma parte (Kögel-Knaber, 2000). Conocer no sólo la cantidad, sino también la composición y tipos de MOS es fundamental a la hora de entender sus funciones así como de comprender los procesos de transformación, mezcla y movilización que le confieren sus características. Por último, la caracterización de la MOS también es de gran ayuda para establecer los posibles orígenes y fuentes de estos materiales.

Los estudios relacionados con el origen, composición y acumulación de materia orgánica en humedales costeros es abundante, sin embargo, la mayoría se centran en la materia orgánica disuelta (Ferrari y Mingazzini, 1995; Lu et al., 2003; Jaffé et al., 2004; Maie et al., 2007; Otero et al., 2007) o en la materia orgánica de sedimentos marinos (Calace et al., 2006; Tremblay y Gagné, 2007). Trabajos similares que traten la caracterización de la materia orgánica en suelos son más escasos.

Ya hace dos décadas, González-Prieto et al. (1989) estudiaron, en el norte de España, las sustancias húmicas presentes en una catena de suelos estuarinos. Estos autores concluyeron que las sustancias húmicas mostraban características tanto terrestres como marinas, y que además, la fracción predominante en la MOS era la comprendida por los ácidos húmicos y fúlvicos. Más lejos de nuestras costas, en el litoral este de Estados Unidos, Alberts, Filips y colaboradores realizaron una serie de investigaciones acerca de SH en marismas colonizadas por *Spartina alterniflora*. Entre las numerosas conclusiones obtenidas destacan las relacionadas con: caracterización espectroscópica de las SH (Alberts et al. 1988; Filip et al., 1991); orígenes de las SH (Alberts et al. 1988; Albert y Filip, 1989); efecto de los microorganismos sobre las SH (Filip y Alberts, 1988, 1993, 1994a); y capacidad de interacción con metales (Alberts y Filip, 1998), minerales (Filip y Alberts, 1994b) y otros compuestos orgánicos (Alberts et al. 1989).

También la influencia de la vegetación en la composición de la MOS ha sido investigada. Goñi y Thomas (2000) estudiaron la materia orgánica en suelos y sedimentos en un transecto bosque -marisma costera, identificando cantidades, composiciones y fuentes de MOS muy diversas en los distintos ambientes a estudio. Más recientemente, Mendonça et al. (2004) caracterizaron ácidos húmicos de una marisma de la Ría de Aveiro (Portugal) por medio de técnicas espectroscópicas. A pesar de sólo comparar un sustrato desnudo con otro vegetado por *Halimione portulacoides* encontraron importantes diferencias, como la mayor labilidad de las SH en la zona vegetada, confirmando así la importancia de la vegetación en las características del sustrato. Sierra et al., (2005) estudiaron, también mediante técnicas espectroscópicas, las SH de ambientes costeros tropicales; y al estudiar los posibles orígenes, encontraron que las SH mostraban procedencias mixtas, tanto terrestres como marinas. Además, demostraron que el grado de influencia de los sistemas terrestres y acuáticos en la composición de las SH dependía, preferentemente, de la localización y fisiografía de los diferentes puntos muestreados.

En relación a los cambios producidos por los procesos antagónicos de reclamado y restauración de marismas, la acumulación y contenido de materia orgánica en los suelos han sido comúnmente usados como indicadores de los grados de degradación o de recuperación de la zona a estudio (Morgan y Short, 2002; Craft et al., 2003; Ellis y Atherton, 2003; Spencer et al., 2008). Sin embargo, los cambios relacionados con las características de esta MOS no han sido frecuentemente tratados. En referencia a este tema puede citarse el trabajo de Craft et al., (1988), donde se utilizaron isótopos estables de C ($\delta^{13}C$) y N ($\delta^{15}N$) para determinar el origen de la MOS en marismas naturales y restauradas. Se observó que, a pesar de que el aporte orgánico predominante era el mismo para ambas y correspondía con la vegetación de marisma, en la marisma restaurada también existían aportes terrestres, presuntamente anteriores al proceso de restauración.

Es por tanto evidente que el estudio de la materia orgánica en suelos y sedimentos de ambientes estuarinos presenta, por si mismo, un gran interés al ser un tema poco tratado hasta el momento. En la presente Memoria Doctoral la caracterización cualitativa de la MOS mediante una amplia gama de técnicas analíticas (Ej.: análisis elemental, espectroscopía de infrarrojo, espectroscopía de UV-visible y de fluorescencia, pirólisis analítica, resonancia magnética nuclear de ^{13}C) y el uso de técnicas novedosas en este tipo de estudios (Ej.: espectroscopía de fluorescencia en tres dimensiones combinada con análisis factorial paralelo) confieren un mayor interés al trabajo así como una mayor consistencia a los resultados obtenidos. Por otra parte, en esta memoria se ha querido también profundizar en algunos de los numerosos factores que condicionan las características de la MOS en los ambientes estuarinos, desde los posibles orígenes o la vegetación, hasta las características geoquímicas del ambiente o procesos antrópicos como el reclamado o la regeneración de marismas. Los objetivos concretos de esta memoria de Tesis Doctoral serán detallados en el correspondiente apartado (sección 5. de este mismo capítulo).

4.- Área de estudio

A continuación se presenta una breve descripción de los sistemas estuarinos que han sido utilizados como zonas de muestreo en los diferentes capítulos de esta tesis doctoral (Figura 1).

Todos ellos están localizados en la costa noroeste de la Península Ibérica, por lo se encuentran bajo la influencia de un clima oceánico templado-húmedo, con temperaturas medias de entre unos 12 y 14º C, sin una amplitud térmica pronunciada, y precipitaciones anuales totales de unos 900-1400 mm.

Figura 1. Localización de los sistemas estuarinos estudiados.

4.1. Estuario de Urdaibai

El estuario de Urdaibai, en la provincia de Vizcaya, representa el eje de la Reserva de la Biosfera de Urdaibai, declarada por la UNESCO en el año 1984. Además, cuenta con otras figuras de protección: ZEPA (Zona de Especial Protección para las Aves), Ramsar (Humedales de Importancia Internacional de la convención Ramsar), LIC (Lugar de Importancia Comunitaria) e IBA (Área Importante para las Aves).

La cuenca hidrográfica que drena el estuario de Urdaibai ocupa una superficie de 183 km^2, siendo la cuenca del río Oka su principal aporte. El estuario tiene una longitud de 12 km, forma de embudo y una anchura más o menos homogénea de 1 km, llegando a 1.5 km a la altura de algunos brazos laterales ligados a pequeñas desembocaduras. El estuario presenta un carácter estratificado en invierno, mientras que en verano existe una mezcla total (Cearreta et al, 2004).

La superficie ocupada por el estuario, 1200 ha, alberga una gran biodiversidad. Se trata del estuario de mayor valor botánico del País Vasco y uno de los más ricos y diversos de todo el litoral cantábrico gracias a las amplias extensiones de marismas que se conservan (Indurot, 2006a). Además, en él se encuentran muchas de las especies de flora estuarina protegida del País Vasco. Por otra parte, Urdaibai representa uno de los humedales más importantes del Cantábrico para el paso migratorio de aves.

Pese a su alto valor ambiental la acción humana ha modificado notablemente sus condiciones naturales. Las presiones antrópicas más importantes se relacionan con la demanda de marismas para zonas de pasto y cultivo, llegando la superficie reclamada a alcanzar el 60% del estuario (Gogeascoechea y Juaristi, 1997). A este aprovechamiento agropecuario hay que sumar otros impactos derivados de actividades industriales e impactos del reciente desarrollo urbanístico.

Actualmente, las marismas ocupan unas 458 ha, y el 75 % de esta superficie se encuentra aún alterada por la presencia de antiguos diques. Sin embargo, debido a su abandono, parte de los terrenos reclamados se están recuperando, aunque este proceso se encuentra todavía en fase inicial.

4.2. Estuario de Villaviciosa

El estuario de Villaviciosa se localiza en la costa centro-oriental de Asturias. La cuenca fluvial que drena este estuario abarca unos 160 km^2 y en él desembocan dos pequeños ríos, el Valdediós y el Nebla, y algunos arroyos. El estuario, tiene una superficie de 700 ha, una longitud de 11 km y una anchura media de unos 1.1 km, que aumenta notablemente en las ensenadas laterales. El reducido aporte fluvial que recibe este estuario le confiere su carácter mesomareal (Flor et al., 1996), diferenciándolo de la mayoría de los estuarios cantábricos que se caracterizan por presentar una mayor influencia fluvial.

El estuario de Villaviciosa ha sufrido grandes cambios debido al reclamado de gran parte de sus suelos estuarinos, ya desde el siglo XIX, cuando los humedales del estuario empezaron a ser desecados para usos

agrícolas y ganaderos. El medio natural en el estuario ha sido, por tanto, ampliamente transformado, y en la actualidad las zonas reclamadas ocupan mayor superficie que los humedales naturales (Fernández y Bueno, 1997). Pese a su alto grado de antropización este estuario posee un gran interés natural, contando con varias figuras de protección, entre ellas las de ZEPA y Reserva Natural Parcial. Estudios botánicos previos le dan una valoración ambiental muy alta, clasificándolo como el primer estuario asturiano en valor botánico por la calidad, diversidad y originalidad de sus marismas remanentes (Bueno, 1996). La mayoría de sus comunidades vegetales están englobadas en la Directiva Hábitat. Las características de la vegetación estuarina existente son únicas en la región, destacando la gran representación de comunidades vegetales halófilas consideradas como hábitats prioritarios (Fernández y Bueno, 1997).

El interés faunístico también es muy elevado por el uso que le dan las poblaciones de aves acuáticas como área de invernada y escala de pasos migratorios. La presencia de algunas especies nidificadoras y las comunidades de invertebrados marinos aportan un valor de importancia añadida (Arce, 1996).

4.3. Estuario de Foz

Este estuario, que forma parte de las Rías Altas Gallegas, se sitúa en la desembocadura del río Masma, confinado por una barrera arenosa actualmente artificial. En conjunto, toda la red drena una superficie de 291 km^2 y la superficie estuarina comprende unas 460 ha. La longitud del estuario es de unos 6 km y su anchura variable, con 1.1 km en la bocana, aumentando en los brazos laterales y estrechándose tierra adentro.

A diferencia de otros grandes estuarios cantábricos, como el de Urdaibai o el de Villaviciosa, el estuario de Foz no ha sufrido un intenso reclamado para fines agropecuarios y la superficie alterada sólo comprende el 6 % de la superficie estuarina total. Las principales modificaciones antrópicas están relacionadas con la canalización de ambos márgenes y alteraciones morfológicas en la zona portuaria de Foz (Indurot, 2006b).

El estuario de Foz se encuentra en excelente estado de conservación, lo que junto con sus características naturales, ha determinado su declaración como LIC, IBA y, a nivel regional, como Espacio Natural en Régimen de Protección General de Galicia. Este estuario posee amplias superficies intermareales y zonas de marisma bien conservadas, en las que se desarrolla una vegetación de gran interés. Desde el punto de vista faunístico presenta también un gran interés ya que acoge numerosas especies de aves, principalmente durante el invierno, representando un importante lugar de paso para las aves migratorias.

4.4. Ría de Ortigueira

La ría de Ortigueira, también denominada Complejo Ortigueira-Ladrido, se localiza en al norte de la provincia de la Coruña, quedando delimitada por Estaca de Bares al este y Cabo Ortegal al oeste. La red hidrográfica que drena la ría está constituida por nueve cursos de agua, de los cuales el río Mera es el de mayor entidad, con una cuenca de drenaje de 127 km^2. La ría principal, la de Ortigueira, tiene forma de *S*, una longitud de unos 9 km y una anchura de hasta 2.5 km. La ría de Ladrido, más pequeña pero con una gran extensión de marismas, no llega a los 3 km de longitud. La superficie total del complejo es de 2920 ha, de las cuales 1663 ha corresponden a las zonas de masas de agua y zona intermareal, mientras que 590 ha son cultivos y 667 ha superficie forestal (Troya y Bernués, 1990).

Esta ría, de carácter mesomareal, comprende extensas llanuras intermareales fango-arenosas, amplias marismas y un considerable sistema dunar. Alberga un conjunto de especies y hábitats muy diverso que le ha llevado a la obtención de varias figuras de protección: ZEPA, Zona Ramsar, Refugio de caza, Espacio Natural en Régimen de Protección General de Galicia e IBA. Además posee un elevado valor como zona de invernada de aves marinas, principalmente anátidas y fochas (de Souza y Lorenzo, 1991).

En la costa atlántica de España el reclamado de zonas estuarinas no es un proceso tan generalizado como en la costa cantábrica, por lo

que las zonas reclamadas son escasas en Ortigueira. Los mayores impactos antrópicos en la ría se deben a: la carretera que la cruza en varios tramos de la zona interna; los impactos indirectos derivados de la cercanía de núcleos poblacionales; y los ocasionados por el marisqueo. También es importante resaltar los vertidos de lodos con altos niveles de Cr y Ni a las marismas, procedentes de una cantera de dunita a pocos kilómetros río Landoi arriba (Otero et al., 2000).

4.5. Laguna Costera de Caldebarcos

En el litoral oeste de A Coruña, el crecimiento de una extensa flecha arenosa en la desembocadura del río Valdebois ha originado uno de los espacios naturales de mayor interés ecológico y paisajístico de Galicia: el sistema dunar de Carnota y la laguna costera de Caldebarcos. Este sistema de barra-laguna litoral ocupa unas 300 ha, y está compuesto principalmente por dos áreas de marisma que se encuentran separadas del mar por una extensa barra litoral arenosa. En el área confluye una serie de pequeños lechos fluviales, siendo el principal el río Valdebois con una cuenca hidrográfica de 1696 ha. Su longitud es aproximadamente de 5.6 km con una anchura que alcanza los 3.4 km.

En cuanto a figuras de protección, pertenece al LIC Carnota-Montes do Pindo, y también cuenta con la denominación de ZEPA y ZEPVN (Zona de Especial Protección de los Valores Naturales). Su importancia ambiental es elevada, ya que presenta un excelente estado de conservación, representando uno de los arenales más importantes de Galicia y es, además, una importante zona de cría e invernada de limícolas y aves marinas.

5.- Objetivos

Los objetivos básicos establecidos para la realización de la presente Memoria de Tesis Doctoral son los siguientes:

I.- Contribuir al conocimiento de los suelos y sedimentos de ambientes estuarinos, prestando especial atención a la acumulación y composición de la materia orgánica en los mismos (Capítulos del I al VI).

II.- Caracterizar la materia orgánica presente en suelos y sedimentos de ambientes estuarinos mediante diversas técnicas analíticas (Capítulos del II al VI).

III.- Caracterizar, mediante técnicas espectroscópicas, fracciones específicas de la materia orgánica de suelos y sedimentos de ambientes estuarinos (Capítulos del II al VI).

IV.- Analizar la influencia de la vegetación en la composición de la materia orgánica edáfica (Capítulos del II al V).

V.- Analizar la influencia de parámetros ambientales, distintos a la vegetación, en la composición de la materia orgánica edáfica (Capítulos III y IV).

VI.- Analizar la influencia de los procesos antrópicos de reclamado en la acumulación y las características de la materia orgánica de suelos y sedimentos estuarinos (Capítulos II y V).

VII.- Analizar la influencia de la regeneración de zonas reclamadas en la acumulación y las características de la materia orgánica de suelos y sedimentos estuarinos (Capítulo V).

VIII.- Proponer nuevas metodologías para la caracterización de sustancias húmicas de suelos y sedimentos (Capítulo VI).

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Capítulo II. Variaciones del stock de carbono orgánico en suelos estuarinos reclamados (Estuario de Villaviciosa, NO de España)

Variations of organic carbon stock in reclaimed estuarine soils (Villaviciosa Estuary, NW Spain)

Resumen

En este capítulo se analiza, en el estuario de Villaviciosa (Asturias, NO de España), el efecto de la polderización en las propiedades edáficas así como en el contenido de carbono orgánico (CO) de los suelos. Los resultados muestran que los suelos polderizados son más ácidos y presentan un menor contenido de carbonatos y una mayor acumulación de CO que los suelos naturales. El stock de CO en los suelos reclamados varía desde 83.2 a 91.8 t ha^{-1}, mientras que en los suelos naturales es de aproximadamente 43.7 t ha^{-1}. Por otra parte, el grado de humificación de los ácidos húmicos extraídos de la capa superficial del suelo es mayor en los suelos reclamados que en los naturales.

Palabras clave: estuario, polderización, carbono orgánico edáfico, edafización.

Abstract

In this chapter a study is carried out to determine the effect of polderization on soil properties and soil organic carbon content in the Villaviciosa Estuary (Asturias, NW Spain). The results showed that the polderized soils were more acidic and contained less carbonates and more soil organic carbon than natural soils. The organic carbon stock in the reclaimed soils ranged from 83.2 to 91.8 t ha^{-1}, whereas in natural soils the level was approximately 43.7 t ha^{-1}. The degree of humification of the surface humic acids also indicated that the stability and degree of decomposition of the organic matter was higher in the reclaimed soils than in natural soils.

Keywords: estuary, polderization, soil organic carbon, soil ripening.

1.- Introduction

The reclamation of estuarine areas has been common practice along much of the European coast. This type of transformation can give rise to substantial changes in the composition and properties of the affected soils. In Spain, the surface area of coastal wetlands has been reduced by nearly half in the last 200 years, mainly as a result of reclamation of the areas for agricultural purposes, grazing and urban development. Transformation of the Villaviciosa Estuary (Asturias, Bay of Biscay, NW Spain) began during the second half of the 19th century. At present, the polderized area comprises a larger area than that of the natural wetlands.

The aim of the present study was to evaluate the effects induced by polderization of soils in the Villaviciosa Estuary, focusing on the changes in the soil carbon stock.

2.- Material and methods

Soil samples were taken on the basis of the morphodynamic zonation of the estuary according to the criteria proposed by Fernández and Marquínez (2002). The zonation (Fig. 1) distinguishes various levels of terrace located at different heights and with varying frequency of flooding by tides: i) Intertidal Flats (IF): areas outside the polderized zones that are subject to daily flooding, with either little or no vegetation; ii) Low Terraces (LT), inside the polderized areas, but notably affected by tides; iii) High Terraces (HT), inside the polderized areas and less affected by tides than the LT.

A total of 10 profiles were selected for study: 2 HT profiles (polderized soils), 4 LT profiles (polderized soils) and 4 IF profiles (natural soils). The following parameters were analysed: (a) pH (soil-water ratio 1:2.5); (b) oxidation pH (soil-solution ratio 1:20, 15% H_2O_2 to pH 5.5, measured after 48 h); (c) total sulphur, nitrogen and carbon (using a LECO CNS-1000 analyser); (d) carbonate content (organic carbon was eliminated by combustion (4 hours at 450º C, Cambardella et al., 2001),

and the carbon content was then determined using the LECO CNS-1000 analyser); (e) total organic carbon (i.e. the difference between the contents of total carbon content and the carbonate).

The contents of oxyhydroxides-Fe and pyrite-Fe were determined in fresh samples; the former were extracted by the citrate-dithionite method and the latter with HNO_3 (for further details see Santín, 2005).

Humic Acids (HA) were extracted from surface soil samples using the IHSS method. Fluorescence emission spectra of HA were measured on a Perkin Elmer LS-50B spectrophotometer (λ_{exc} = 240 nm, from 260 to 600 nm.). The degree of humification of HA was calculated from these spectra using the method proposed by Zsolnay (1999).

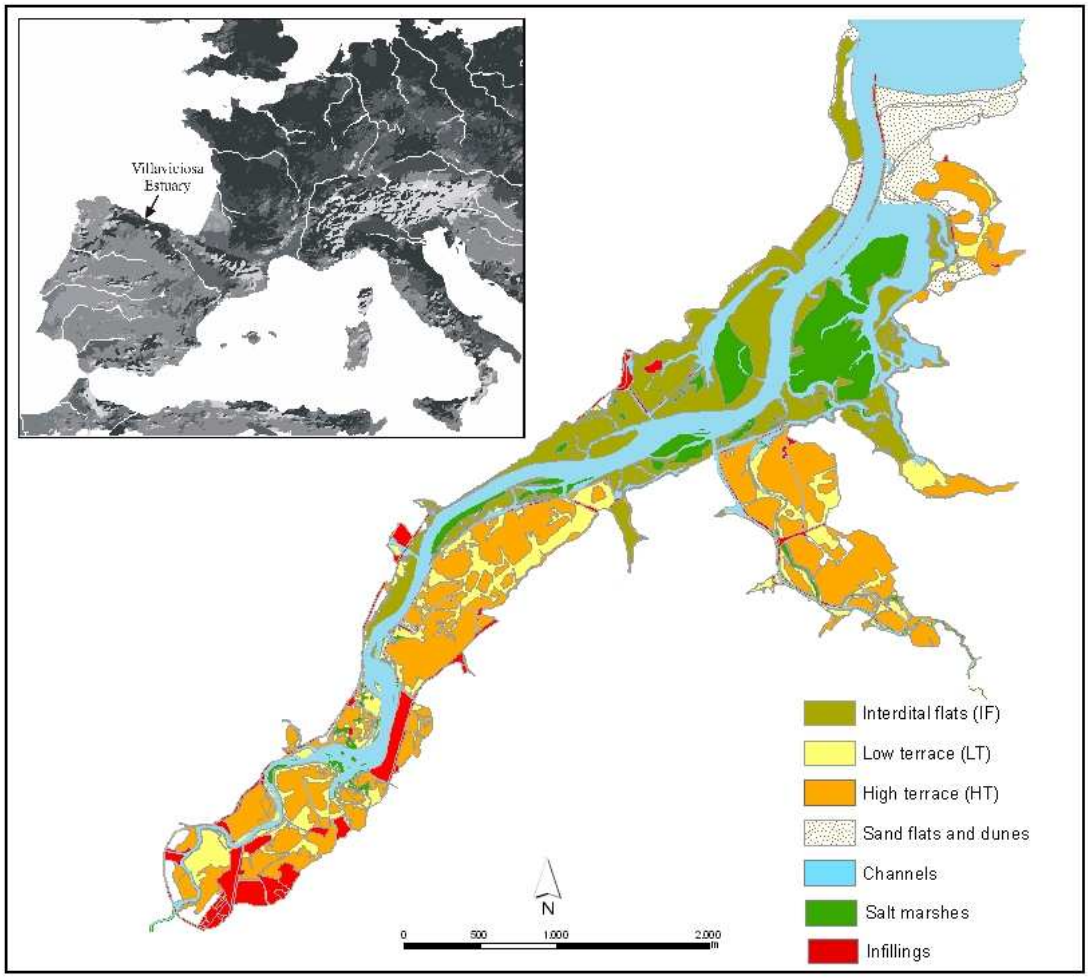

Figure 1. Morphodynamic zoning in the Villaviciosa Estuary.

3.- Results and discussion

3.1. Effects of polderization on soil properties

As a result of polderization, part of the intertidal flats became LT and HT, where the frequency of flooding is lower. One of the main consequences of this reclamation process was a decrease in soil pH (Table 1). The lowest pH values (pH 4-5.5) were observed in the upper horizons of the HT. In these soils, the decrease in frequency of flooding gave rise to greater aeration, which in turn led to oxidation of reduced forms of Fe and Mn and metal sulphides, which are characteristics of this type of soil (Otero and Macías, 2002, 2003).

Table 1. Description of soil samples, and parameters analysed.

Profile	Horizon	Depth (cm)	pH	pH ox.	%TC	%TN	%TS	%IC	%TOC
High Terrace (pastures)									
1	Ap	0-5	5.5	2.9	5.40	0.38	0.07	0.15	5.25
	A/C	5-60	6.8	4.5	0.95	0.10	0.05	0.04	0.91
	Cr	>60	6.8	5.9	0.89	0.07	0.88	0.20	0.70
2	Ap	0-10	4.2	2.4	8.63	0.77	0.25	0.45	8.18
	A/C	10-40	5.1	3.7	1.19	0.13	0.03	0.07	1.12
	Cg	>40	7.4	6.4	1.40	0.09	0.45	0.30	1.10
Low Terrace (profiles 3-4:pastures, 5-6: rush communities)									
3	Ap	0-15	6.1	3.4	5.56	0.37	0.19	0.06	5.50
	A/C	15-40	6.6	4.4	2.46	0.18	0.08	0.13	2.33
	Cr	>40	3.5	1.9	1.77	0.13	0.94	0.07	1.70
4	Ap	0-10	7.8	6.6	7.80	0.24	0.20	4.54	3.26
	Cr	>10	8.5	6.9	4.72	0.05	0.32	4.74	n.d
5	Ag	0-20	7.4	4.9	4.29	0.29	0.38	0.32	3.98
	Cr	>20	6.9	3.2	2.01	0.14	1.29	0.19	1.82
6	Ag	0-20	7.5	6.1	3.47	0.22	0.20	0.32	3.15
	Cr	>20	7.8	5.3	1.85	0.15	0.95	0.29	1.56
Intertidal Flat (no vegetation or Zostera nolti)									
7	Ar	0-20	7.7	5.2	1.53	0.10	0.85	0.23	1.30
8	Ar	0-20	7.8	6.2	3.19	0.13	0.78	1.14	2.05
9	Ar	0-20	7.7	5.8	2.49	0.11	0.50	0.80	1.70
10	Ar	0-20	7.8	6.3	3.89	0.17	0.94	1.26	2.63

pH ox. = Oxidation pH; %TC = % Total Carbon; %NT = Total Nitrogen; %ST = Total Sulphur; %IC = Carbonates; %TOC = Total Organic Carbon; n.d.= not determined.

This hypothesis is supported by the results obtained from the Fe fractionation. The results (Fig. 2) revealed large concentrations of pyrite-Fe throughout the soil profiles from the natural area (IF), whereas in the HT, the presence of pyrite-Fe was only observed below 100 cm depth (in pasture land), presumably as a result of shallow oxidation due to polderization of the soil.

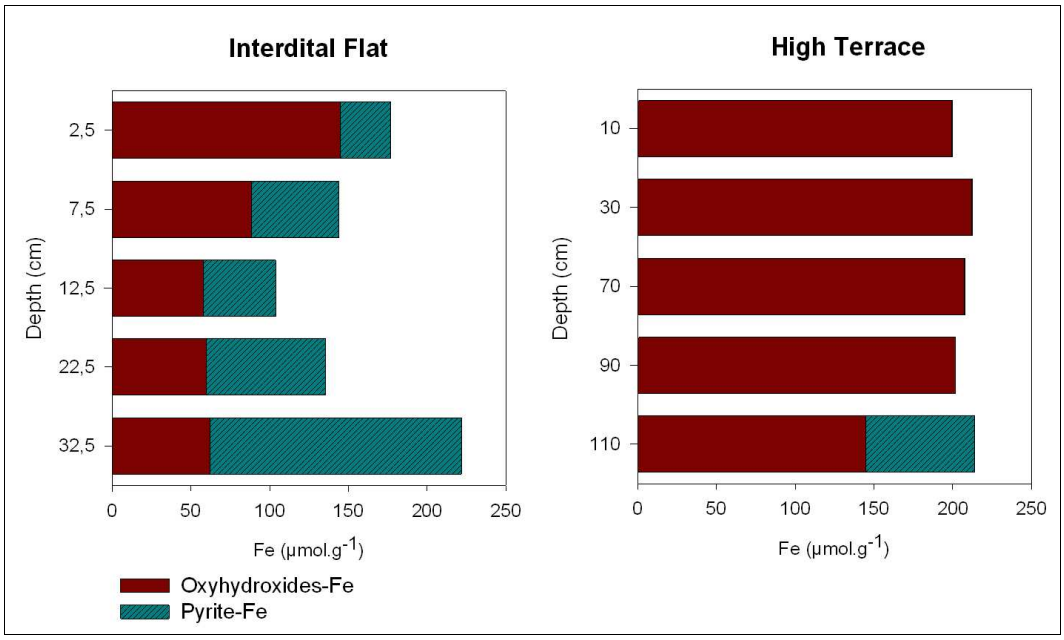

Figure 2. Concentration of Oxyhydroxides-Fe and pyrite-Fe in two soil profiles: in IF under *Zostera noltii* (left) and in HT under pasture (right).

In accordance with the results obtained from the fractionation of the Fe, the oxidation pH <3.5: (Table 1) reflected the existence of sulphuric material in some of the reclaimed soils (profiles 1, 2, 3 and 5) (ISSS-ISRI-FAO, 1998).

The carbonate content was lower in the reclaimed areas than in the intertidal flats (Table 1). The carbonate content generally decreases in polderized soils as a result of dissolution caused by soil acidification. However, this did not occur in profile 4, because it is reclaimed soil over a sand bar with a naturally high carbonate content caused by the presence of bioclastic rock (Fernández et al., 2005).

The TOC and TN were significantly correlated (r^2 = 0.93, N = 19, P<0.001), suggesting an organic origin for most of the N in these soils. However, the TS content was not significantly correlated with the TOC. There was an accumulation of S in the lower horizons of the reclaimed areas, as well as in the intertidal flat (Table 1). In these horizons, in which the TOC was relatively low, S was present as inorganic forms, mainly as iron sulphides (Otero and Macías, 2001). The increase in pyrite-Fe in these horizons appears to confirm this hypothesis (Fig. 2).

3.2. Effect on the soil carbon stock

The TOC content ranged from 0.70% to 8.18% and the highest accumulation occurred in the first few centimetres of the reclaimed soils (Table 1).

The IF soils were incipient soils, without any well-defined horizons. The soil organic carbon accumulation was rather low (Table 1) and subject to a strong tidal influence. On the other hand, reclamation led to a decrease in the frequency of flooding, which caused soil ripening, which in turn allowed settlement of plant communities and resulted in accumulation of a greater amount of TOC in the reclaimed land. The average concentration of TOC in the first 20 cm of the IF soil was 43.7 t ha^{-1} and in the LT and HT soils it was respectively 83.2 t ha^{-1} and 91.8 t ha^{-1}. A preliminary study of the quality of the organic matter in these environments was carried out to confirm the above-mentioned hypothesis. The degree of humification of the humic acids was calculated from the fluorescence emission spectra of surface samples (0-10 cm) from the three morphodynamic units (Fig. 3).

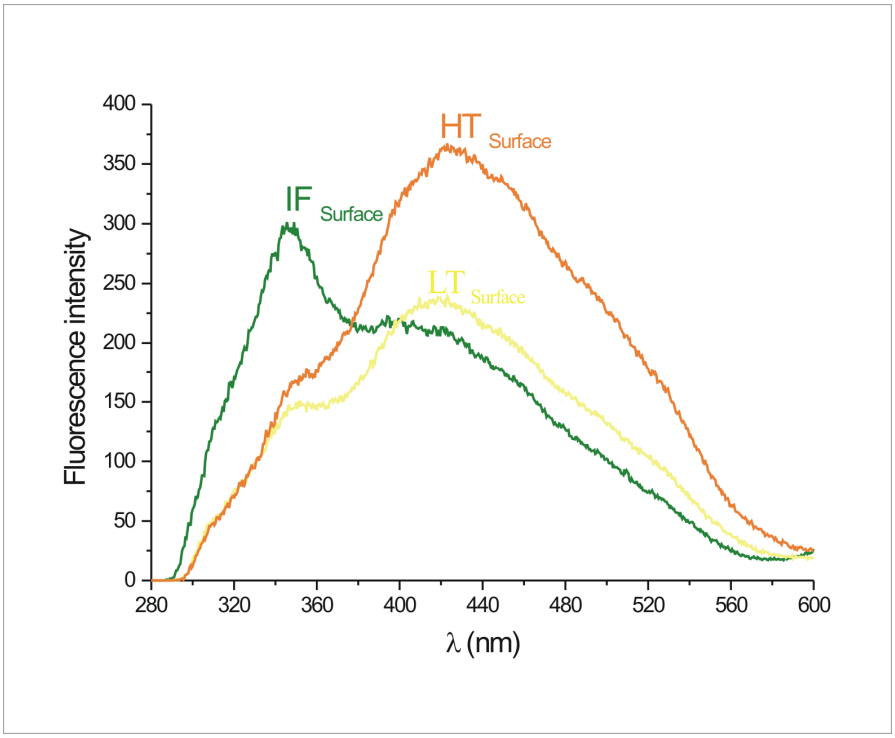

Figure 3. Fluorescence spectra of HA (Emission spectra, λ_{exc} = 240 nm from 260 to 600 nm).

The most humified organic matter corresponded to the HT sample (degree of humification = 0.75), followed by the LT sample (0.49). The lowest degree of humification (0.17), characteristic of less decomposed and more labile soil organic matter, corresponded to the IF soil. Further characterization of the soil organic matter in these environments must be carried out to provide a more complete view of how polderization affects the stock of TOC, not only as regards the amount of organic matter accumulated, but also the composition and quality.

4.- Conclusions

The process of reclamation in the Villaviciosa Estuary has led to transformation of the soils, from incipient intertidal flats to more developed soils, in the lower and higher terraces. The resulting decrease in the frequency of flooding and the increase in drainage and aeration have led to soil ripening, resulting in: differentiation of horizons, acidification, dissolution and loss of carbonates and an increase in the accumulation of organic matter, as well as a higher degree of humification.

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Capítulo III. Caracterización de sustancias húmicas en suelos de marisma con junco marítimo (*Juncus maritimus*)

*Characterization of humic substances in salt marsh soils under sea rush (*Juncus maritimus*)*

Resumen

En este capítulo se describen las características de las sustancias húmicas (SH) de suelos de marismas, analizándose además las relaciones entre la composición de las SH y algunos factores geoquímicos. Para ello se estudiaron tres marismas con la misma vegetación (*Juncus maritimus*) pero con diferentes características geoquímicas. La caracterización cualitativa de las sustancias húmicas edáficas se realizó mediante análisis elemental, espectroscopía de infrarrojo por transformada de Fourier, espectroscopía de fluorescencia y resonancia magnética nuclear de ^{13}C.

Las SH de suelos de marisma con junco marítimo (*Juncus maritimus*) muestras algunas características comunes: bajo grado de humificación, aromaticidad reducida y alta proporción de compuestos lábiles, principalmente polisacáridos y proteínas. Sin embargo, a pesar de que los tres suelos a estudio están sujetos a la influencia del mismo tipo de vegetación, las SH muestran importantes diferencias. Por lo tanto, la composición de las SH está determinada no sólo por la naturaleza del material orgánico original sino también por factores ambientales como la textura del suelo, las condiciones redox o la influencia mareal. En general, un aumento de la humificación se relaciona con condiciones óxicas asociadas a predominio de texturas gruesas en la fracción mineral del suelo, mientras que la preservación de compuestos orgánicos lábiles está asociada a valores inferiores del potencial redox y a texturas edáficas finas.

Palabras clave: sustancias húmicas, suelos de marisma, espectroscopía, *Juncus maritimus*.

Abstract

Humic substances (HS) from salt marsh soils were characterized and the relationships among HS composition and some geochemical factors were analysed. For this, three salt marshes with the same vegetation cover (*Juncus maritimus*), but with different geochemical characteristics, were selected. The qualitative characterization of the soil humic acids and fulvic acids was carried out by elemental analysis, FTIR spectroscopy, fluorescence spectroscopy and VACP/MAS ^{13}C NMR spectroscopy.

HS from salt marsh soils under sea rush (*Juncus maritimus*) displayed some shared characteristics such as low degree of humification, low aromatic content and high proportion of labile compounds, mainly polysaccharides and proteins. However, although the three salt marsh soils under study were covered by the same type of vegetation, the HS showed some important differences. HS composition was found to be determined not only by the nature of the original organic material, but also by environmental factors such as soil texture, redox conditions and tidal influence. In general, an increase in the humification process appeared to be related to aerobic conditions and predominance of sand in the mineral fraction of the soil, while the preservation of labile organic compounds may be associated with low redox potential values and fine soil texture.

Key words: humic substances, salt marsh soils, spectroscopy, *Juncus maritimus*.

1.- Introduction

Net primary production is high in tidal wetlands, particularly salt marshes and mangroves swamps, in which soils and sediments are the main reservoirs of organic carbon (Barnes, 1980) and play an important role in the global carbon cycle (Chmura et al., 2003). The cycling and stabilization of carbon is closely related to the structural characteristics and chemical composition of the organic matter (OM) (Kögel-Knabner, 2000). Humic substances (HS), mainly humic acids (HA) and fulvic acids (FA), are an essential part of soil organic matter (SOM) (Stevenson, 1994) and the nature and stability of these substances affect carbon and nitrogen cycles and carbon sequestration.

Salt marshes are also important sources and sinks for a variety of natural and anthropogenic compounds (Filip and Alberts, 1994a), and humic and fulvic acids play a major role in binding hydrophobic and hydrophilic species such as inorganic contaminants or pesticides (Senesi, 1992; Kordel et al., 1997). Despite the importance of humic substances in the environment, most research concerning these coastal wetland systems has focused on dissolved organic matter (DOM) (Ferrari and Mingazzini, 1995; Lu et al., 2003; Jaffé et al., 2004; Maie et al., 2007; Otero et al., 2007) or OM in marine sediments (Calace et al, 2006; Tremblay and Gagné, 2007), and corresponding studies of salt marsh soils or sediments are scarce.

The influence of salt marsh vegetation on SOM composition has been widely investigated. For example, Alberts et al. (1988) carried out a study of the HS composition of marsh muds in a Georgia salt marsh estuary colonized by *Spartina alterniflora*, and found evidence of sources of humic substances other than *S. alterniflora* debris. Furthermore, Goñi and Thomas (2000) studied the OM contents of soils and sediments along a forest-brackish marsh-salt marsh transect on the south-eastern coast of the US, and observed very different quantities, compositions and sources of OM in the different environments studied. These authors identified the predominance of organic compounds derived from *Juncus* sp. and *Spartina* sp. at the marsh sites. More recently, Mendonça et al. (2004)

characterized sedimentary humic acids from a salt marsh in a lagoon on the Atlantic European coast (Ría de Averio, Portugal) by means of spectroscopic techniques. They compared non-vegetated sediment and other sediments colonized by *Halimione portulacoides*, and concluded that colonizing plants promote incorporation of proteinaceous and polysaccharidic material in the humic acids.

Nevertheless, it is not only the original plant material that determines SOM composition (Stevenson, 1994). The geochemical environment where the vegetation is developed and decomposed is also fundamental (Goñi and Thomas, 2000). Environmental gradients in salt marshes are mainly influenced by physiographical location and flooding regime (Zedler et al., 1999; Otero and Macías, 2001), which affect parameters such as salinity, redox conditions and soil texture (Otero and Macías, 2001, 2003).

In order to investigate the quantity and composition of SOM as well as how it is affected by the geochemical environment, three salt marshes with the same vegetation cover (rush communities), but different geochemical characteristics, were chosen: an estuarine salt marsh, a polder salt marsh and a coastal lagoon. Since the geochemical characteristics can also change throughout the profile, two samples were collected from each site, one at the surface and another at depth.

The aim of the present study was to characterize the HS, i.e., humic and fulvic acids, in salt marsh soils by means of spectroscopic techniques such as FTIR, fluorescence and VACP/MAS ^{13}C NMR spectroscopy, and to establish whether differences in the geochemical environment affect the SOM composition at different sites with the same vegetation cover (sea rush, *Juncus maritimus*).

2.- Material and methods

2.1. Study sites and soil sampling

The study area is located on the northwestern coast of the Iberian Peninsula. The climate is oceanic, with mean annual temperatures

between 12 and 14º C and total annual precipitation between 900 and 1100 mm.

In order to compare how different environmental conditions affect the composition and quality of organic matter, three sites with the same vegetation cover - sea rush communities - but with different physiographical, morphological and tidal-influence characteristics, were selected: i) RI: a rush community in a natural salt marsh, situated at the upper part of the Villaviciosa Estuary; ii) RII: a rush community in a polder, also in the Villaviciosa Estuary. This area was reclaimed 150 years ago but has been recolonized by natural vegetation (i.e. sea rush) since the cessation of agricultural activity. iii) RIII: a rush community located at the upper estuary, a coastal lagoon, with low tidal influence. This highly infilled lagoon is partially closed by a sand barrier and is only flooded during spring tides.

One sampling point was chosen at each site and a soil sample was taken from the upper soil layer, where the influence of the vegetation is higher, and another at depth, where the influence of the vegetation cover is lower (Table 1).

2.2. Soil chemical and physicochemical analyses

In the field, the redox potential (Eh) was measured with an oxidation-reduction potential (ORP) platinum electrode. Final readings were corrected by addition of the potential value (+244mV) of a calomel reference electrode. The pH was measured with a Crisol micropH 2000 with a glass electrode. In dried samples, particle size was determined by the pipette method (Gee and Bauder, 1986). Total C, S, N in the bulk soil and the elemental composition (C, H, N, O, S) of the extracted HA and FA were measured with a LECO CHNS-932 elemental analyzer. Organic carbon was eliminated from the bulk soil by combustion during for 4 hours at 450º C (Cambardella et al., 2001) and the remaining inorganic carbon (IC) was measured. Organic carbon (OC) was then estimated as the difference between total carbon and IC, after prior correction for weight loss.

2.3. Soil organic matter chemical fractionation

Humic acids and fulvic acids were extracted from dried and 2 mm sieved samples, and purified according to the methodology of the International Humic Substances Society (Swift, R.S., 1996). Briefly, the method included extraction of the HS with 0.1 mol L^{-1} NaOH, at a soil:solvent ratio of 1:10. The samples were centrifuged and the HA was then separated from the supernatant by precipitation with 6 mol L^{-1} HCl added to the extract, until pH 2.0. The precipitated HA were separated by centrifugation, purified by dialysis on a Spectrapor membrane (size exclusion limit 6000–8000 D) and freeze-dried.

The FA were isolated from the acid supernatant with an SUPELITE DAX-8 resin at pH 2.0 (Thurman and Malcolm; 1981). The FA were purified by removal of inorganic salts on a Spectrapor dialysis membrane (1000 D). The FA in solution were then freeze-dried.

2.4. Spectroscopic characterization of humic substances

FTIR Spectroscopy

Spectra were recorded on a Fourier-Transformed Infrared Perkin Elmer, Paragon 1000 PC spectrophotometer. The KBr pellets (1 mg HS: 100 mg KBr, spectrometry grade) were obtained by pressing at 10 000 kg cm^{-2} for 2 min (Stevenson, 1994). The spectral resolution was 4 cm^{-1}; the means of 64 scans were obtained to reduce noise.

Fluorescence Spectroscopy

Fluorescence experiments were carried out with humic substances dissolved in aqueous solutions of $NaHCO_3$ (0.05 $molL^{-1}$), at a concentration of 20 mg L^{-1} and pH of approximately 8. These conditions were determined as optimal in a previous study (Milori et al., 2002). Spectra were recorded by a Perkin Elmer LS 50B luminescence spectrometer at a scan speed of 200 nm min^{-1}. For HA, the emission and excitation slits were set at 10 nm; for FA the slits were reduced to 7 nm.

Emission spectra were measured at an excitation wavelength of 240 nm over a range of 300 to 600 nm. For FA, synchronous spectra were

also recorded over a range of 200 to 600 nm by simultaneous scanning of the excitation and emission wavelengths at a constant wavelength difference (Δλ = 18 nm).

Humification indexes were estimated from emission spectra according to Zsolnay et al., 1999, and from synchronous spectra according to Kalbitz et al., 2000.

^{13}C VACP/MAS NMR Spectroscopy

^{13}C variable amplitude cross polarization magic angle spinning (VACP/MAS) nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DSX 200 spectrometer operated at a ^{13}C resonance frequency of 50.32 MHz. The samples were confined in a zirconium oxide rotor with an external diameter of 7 mm. The VACP/MAS technique was applied with a contact time of 1 ms, a spinning speed of 6.8 kHz and a pulse delay of 1 s. For quantification, the spectra were subdivided into different chemical shift regions according to Knicker and Lüdemann (1995): alkyl C (0–45 ppm), N-alkyl/methoxyl C (45-60 ppm), O-alkyl C (60–110 ppm), aromatic C (110–160 ppm), carboxyl C (160-185 ppm) and carbonyl C (185–245 ppm). The relative ^{13}C intensity distribution was determined by integrating the signal intensity in different chemical shift regions with an integration routine supplied with the instrument software. Corrections concerning the intensity distribution of spinning side bands were made (Knicker et al., 2005). These data were used to calculate the degree of aromaticity (%) (aromatic C * 100 / (alkyl C + N-alkyl/methoxyl C + O-alkyl C + aromatic C)) (Hatcher et al., 1981). The alkyl/O-alkyl C ratio was also calculated as an indicator of the degree of SOM decomposition (Baldock et al., 1997).

3.- Results

3.1. General characterization of soils

Some descriptor parameters of the studied soils are shown in Table 1. Although the vegetation cover was the same at the three studied sites, the physiographical and geochemical characteristics differed. The main

difference was in the frequency of flooding, which was low at the lagoon site (RIII), only affected by spring tides, and higher at the other two sites (RI and RII), both flooded several times each season. The soil texture varied among sites and also at depth in the same profile (Table 1). This high variability is typical of this type of soil in sedimentary environments (Bridges, 1997; Otero and Macías, 2003). The sandiest soil was RIII, with 52.8 % of sand at surface and 90.5 % at depth. The RI soil had the finest grain size, with only 3.6 % of sand at surface and 21.4 % at depth. The sand content increased with depth in the three soils (Table 1).

The OC content was higher in the surface layer (32-239 $mg.g^{-1}$) than in the deeper layer (5-24 $mg.g^{-1}$). The greatest accumulation of OC was observed in RII at the surface, where the low Eh combined with the fine texture may help to protect and stabilize the SOM (D'Angelo and Reddy, 1999; Tremblay and Gagné, 2007). The IC content was low in all samples (< 10 $mg.g^{-1}$) and the pH did not vary greatly (between pH 6.5 and 7.6) (Table 1).

Table 1: Descriptor parameters of soil samples.

	Villaviciosa Estuary (5º 24' W 43º 31' N)				*Caldebarcos lagoon (9º 5' W 42º 50' N)*	
	Natural Rush (RI)		Polder Rush (RII)		Lagoon Rush (RIII)	
	$RI_{surface}$	RI_{deep}	$RII_{surface}$	RII_{deep}	$RIII_{surface}$	$RIII_{deep}$
Depth (cm)	0-5	40-50	0-10	50-70	0-20	90-100
OC ($mg.g^{-1}$)	156	24	239	6	32	5
IC ($mg.g^{-1}$)	2.6	0.7	9.4	1.6	1.1	2.1
pH	6.9	6.5	7	6.8	7.4	7.6
Eh (mV)	372	273	108	n.d	384	114
Sand (%)	3.6	21.4	7.3	38.1	52.8	90.5
Silt (%)	54.3	27.3	33.5	18.9	27.2	4.3
Clay (%)	42.1	51.3	59.2	43	20	5.2
Frequency of flooding	high		medium		low	

n.d: not determined

3.2. Elemental composition

The elemental composition and atomic ratios of HA and FA are shown in Table 2. The HA contained more C (HA 51.4 – 52.6 %, FA 43.7 – 46.3 %) and N (HA 4.4 – 5.7 %, FA 3.2 – 4.9) than the FA. However, the FA contained higher proportions of H (6.0 – 6.9 %) and O (41.3 – 45.1 %). The amount of S was more variable in the FA (contents of 0.3 – 2.0 %)

than in the HA (1.1-1.5%). In the FA, the S content showed a clear tendency to increase with depth in the three profiles. Enrichment of S in humic substances, especially in FA, has been observed in anoxic environments via reaction of reduced sulphur, mainly H_2S, with organic matter to release several types of organosulphur compounds (Ferdelman et al., 1991; Brüchert and Pratt, 1996).

The high atomic ratios of H/C (1.1 – 1.4 for HA and 1.6-1.9 for FA) indicate a predominant aliphatic character for both HA and FA, whereas the O/C ratios, 0.5 in HA and between 0.7 and 0.8 in the FA, suggest a relatively large amount of O-containing groups in these HS (Senesi et al., 2003).

Table 2. Elemental composition (ash-free, expressed as percentages) and atomic ratios of humic and fulvic acids.

Sample	C	H	N	S	O	C/N	H/C	O/C
Humic Acids								
$RI_{surface}$	52.6	5.5	4.7	1.2	36.0	13.2	1.3	0.5
RI_{deep}	51.5	5.6	4.8	1.2	36.9	12.4	1.3	0.5
$RII_{surface}$	51.4	5.9	5.7	1.5	35.4	10.5	1.4	0.5
RII_{deep}	51.9	5.9	5.6	1.3	35.3	10.9	1.4	0.5
$RIII_{surface}$	52.6	4.8	4.4	1.1	37.1	13.8	1.1	0.5
$RIII_{deep}$	52.5	5.3	4.9	1.2	36.0	12.5	1.2	0.5
Fulvic Acids								
$RI_{surface}$	45.1	6.6	4.9	0.3	43.1	10.8	1.8	0.7
RI_{deep}	44.9	6.0	3.5	0.6	45.1	14.8	1.6	0.8
$RII_{surface}$	44.6	6.1	3.9	1.2	44.1	13.2	1.6	0.7
RII_{deep}	43.7	6.9	3.2	1.8	44.3	15.9	1.9	0.8
$RIII_{surface}$	45.1	6.1	3.3	0.9	44.6	15.7	1.6	0.7
$RIII_{deep}$	46.3	6.8	3.6	2.0	41.3	14.9	1.8	0.7

The C/N ratio was low in all samples (10.5-13.8 for HA and 10.8-15.9 for FA). These values were lower than those typically attributed to upland soil HS (e.g. Senesi et al., 2003), slightly higher than those recorded for HS in aquatic sediments (e.g. Kappler et al., 2001; Miserocchi et al., 2007), and similar to those reported for salt marsh sediments (e.g. Alberts and Filip, 1989; Mendonça et al., 2004). Furthermore, there was no common trend in the variation in the C/N ratio with depth. The value of this ratio only increased with depth in the FA from the RI and RII soils (Table 2), as previously found for humic substances in similar

environments (Filip and Alberts, 1994b; Mendonça et al., 2004; Calace et al., 2006). However the C/N ratio decreased at depth in the HA from RI and RIII and in the FA from RIII, which suggests immobilization of N during humification (Chabbi and Rumpel, 2004) perhaps due to the incorporation of microbial N-rich compounds into HS (Klap et al., 1999) or to the stabilization of N-rich compounds by different processes such as chemical protection or interaction with the soil mineral phase (Knicker, 2004).

3.3. FTIR spectroscopy

Identification of the main absorption bands was based on data published by Silverstein et al. (1991) and Stevenson (1994). Differences in the relative intensities of bands can be appreciated between each FA and the corresponding HA (Figure 1). Firstly, the intensity of the broad band at 3400 cm^{-1}, attributed to O-H and N-H stretching, was higher in FA, which suggests the presence of important amounts of these groups in the HS. Secondly, in the HA, the higher signal intensities at 2930 cm^{-1} and 2850 cm^{-1} (asymmetric and symmetric stretching of methylene groups), and also a signal at 2960 cm^{-1} (asymmetric stretching of methyl groups) indicate a higher aliphatic C content than in FA.

Moreover, in the HA and FA spectra most of the carboxyl groups were in the dissociated form. The peak attributed to COO^- asymmetric stretching can be clearly observed in the HA spectra at 1650 cm^{-1}. However, for FA spectra this peak appears to overlap with the amide II band (1600 cm^{-1}), although the peak assigned to the COO^- symmetric stretching can be observed at 1400-1380 cm^{-1}. Furthermore, the signal at 1715 cm^{-1}, attributed to C=O stretching of COOH and ketones, did not appear in the FA spectra and only appeared as a shoulder in the HA spectra. The peak at 1260-1200 cm^{-1} (C–O stretching and OH deformation of COOH) was also more intense in the HA than in the FA spectra.

The presence of protein-like compounds was revealed by two peaks, one at 1660-1630 cm^{-1}, assigned, among others, to C=O stretching of amide groups, and another at 1540-1510 cm^{-1} , assigned to N-H deformation and C=N stretching of amide groups. These two peaks were only distinguished in the HA spectra.

Finally, in all spectra, a peak was detected at 1080-1030 cm^{-1} and ascribed to the C-O stretching of polysaccharides and/or to Si-O vibrations of silicate impurities.

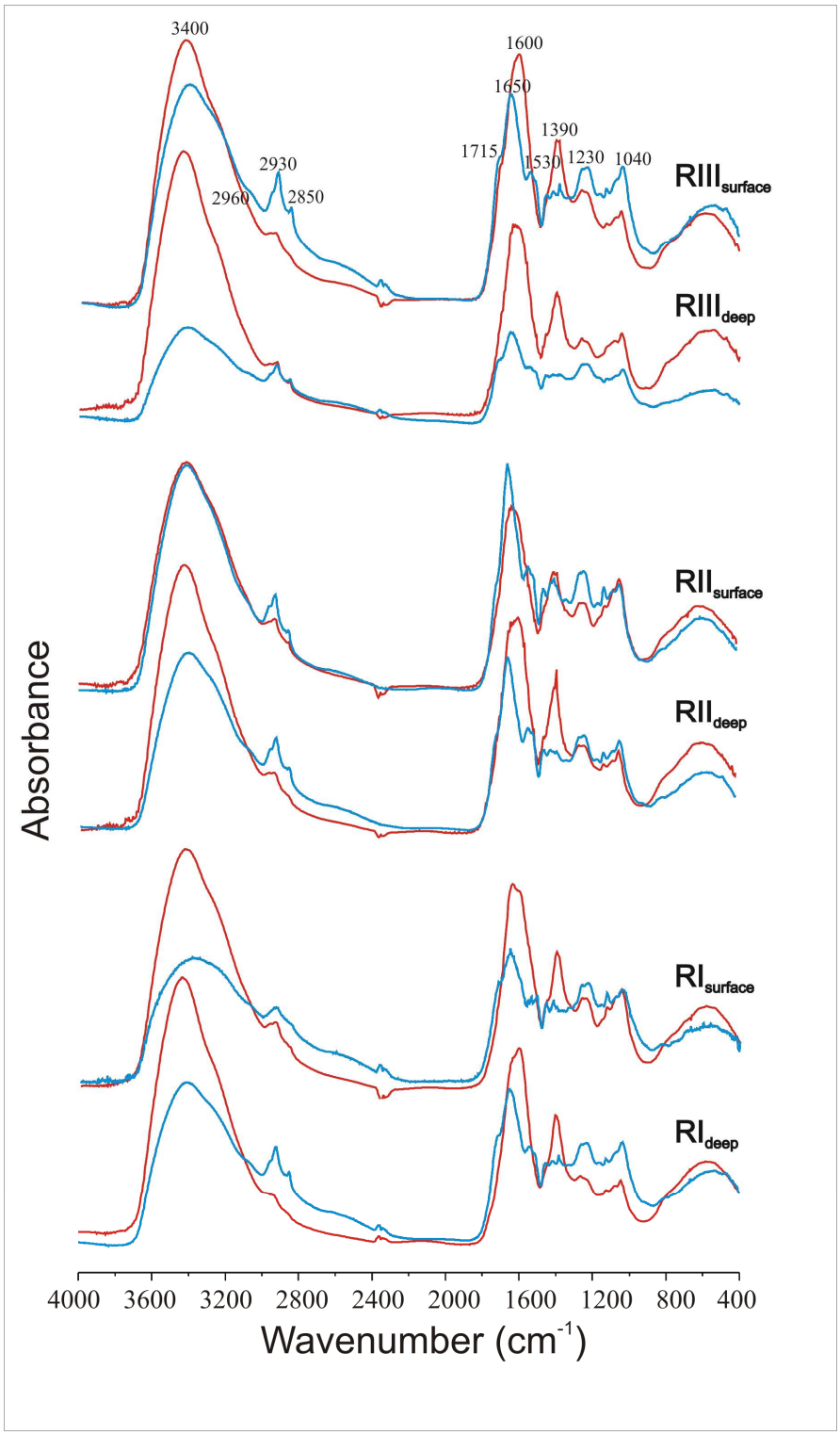

Figure 1. FTIR spectra for humic acids (blue line) and fulvic acids (red line) from the three soils under *Juncus maritimus*.

Comparison of the HS spectra in different environments revealed that HA from the lagoon surface ($RIII_{surface}$) contained the greatest amount of aliphatic C and the surface sample of the natural rush ($RI_{surface}$) the lowest amount, whereas there were no important differences among sites in the HA in deep layers. FA composition was very similar in the soils from the three sites. In the surface layer the only difference was a lower polysaccharide content in RIII than in RI and RII. The three FA spectra for the deep samples were almost identical (Figure 1).

The variations in each profile did not follow the same pattern for the three sites. In the RI soil there was a slight increase in the aliphatic compounds at depth in the HA spectra and a decrease in the COOH signal and polysaccharides in the FA spectra; in the RII soil, the intensity of the signal at 1400 cm^{-1} (OH deformation and C-O stretching of carboxylic acid) for the HA spectra was higher at surface, and in the FA spectra there was a increase in COO^- signal at depth. In the RIII soil the FA in the deep layer contained more polysaccharides than the FA in the surface layer.

3.4. Fluorescence spectroscopy

Emission spectra

The HA emission spectra (Figure 2) presented one main band, with the maximum intensity located at 418 nm for the RI and RII surface samples, and at 430 nm for RI and RII deep samples, but at longer wavelengths in the RIII HA (at 461 nm for $RIII_{surface}$ and at 455 nm for $RIII_{deep}$). Moreover, a shoulder was detected at 350 nm in all the HA spectra, and was particularly intense in $RI_{surface}$, $RII_{surface}$ and RII_{deep}. This shoulder may indicate the existence of protein-like fluorophores and/or phenolic moieties in the HA. Maie et al. (2007) attributed the excitation/emission maximum at 280/325 nm in DOM in estuarine ecosystems to these compounds.

The humification index calculated from the HA emission spectra (Zsolnay et al., 1999) is shown in Table 3. The most humified HA were

found in RIII, in both the surface (2.05) and the deep (1.31) layer. Furthermore, the degree of humification did not follow the same trend with depth in the three sites. In RI, the Zsolnay index increased from 0.39 at the surface, to 0.83 at depth, but decreased in RIII, and in RII there was no significant variation with depth (Table 3).

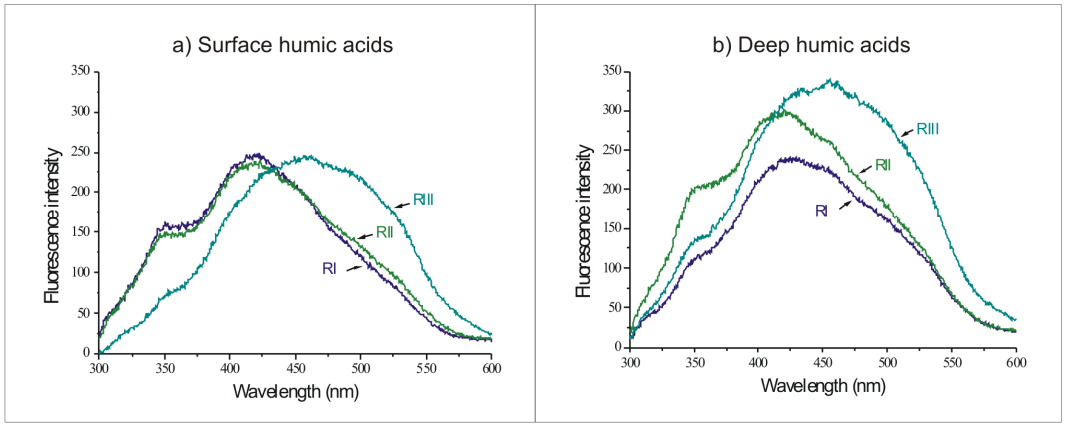

Figure 2. Fluorescence emission spectra of soil humic acids (λ_{exc} = 240 nm). (a) Surface samples. (b) Deep samples.

As regards the FA emission spectra, all were similar, with a single broad band of maximum intensity at 421±3 nm and no shoulder at shorter wavelengths (figure not shown). The Zsolnay index showed an increase in the degree of humification at depth in the three soils (Table 3).

Table 3. Humification indexes (HI) for HA and FA determined by fluorescence spectroscopy.

	Humic acids	Fulvic acids	
Sample	$HI_{Zsolnay}$	$HI_{Zsolnay}$	$HI_{Kalbitz}$
$RI_{surface}$	0.39	0.37	0.31
RI_{deep}	0.83	0.75	0.83
$RII_{surface}$	0.49	0.42	0.39
RII_{deep}	0.49	0.55	0.61
$RIII_{surface}$	2.06	0.53	0.50
$RIII_{deep}$	1.31	0.62	0.70

Generally, soil FA present the maximum intensity at lower wavelengths than their HA counterparts (Senesi et al., 1991; Fuentes et al., 2006). However, this was not observed in the soils under study.

Substantial differences between HA and FA were only observed in the lagoon soil (RIII), where the more humified HA were present. Furthermore, in all the soils studied the maxima for the HS emission spectra, both HA and FA, were lower than those attributed to HS from upland soils (Senesi et al., 1991; Zsolnay et al., 1999; Milori et al., 2002), but were consistent with those attributed to aquatic natural organic matter (Chen et al., 2002) or other fresh organic materials (Senesi et al., 1991).

Synchronous spectra

The FA synchronous spectra are displayed in Figure 3. Three main peaks were identified in all of them. Peak I (281-288 nm) is associated with the presence of proteinaceous materials and mono-aromatic compounds (Ferrari and Mingazzini, 1995). These organic compounds are usually found in DOM, mangrove and sawgrass leachates, and aquatic FA (Jaffé et al., 2004; Lu et al., 2003, Lu and Jaffé, 2001, De Souza Sierra et al., 1994), but not in soil FA (Shin et al., 2001).

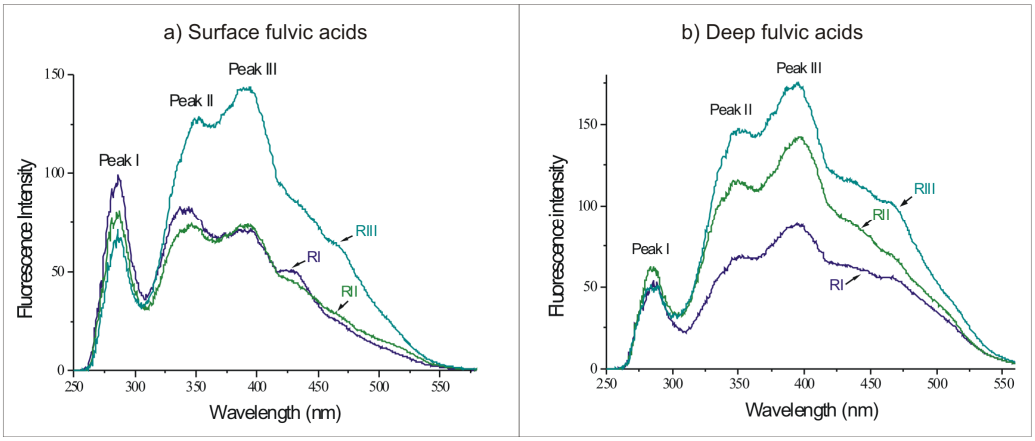

Figure 3. Fluorescence synchronous spectra of soil fulvic acids (Δλ = 18 nm). (a) Surface samples. (b) Deep samples.

The other two peaks, peak II (345-355 nm) and peak III (385-395 nm), indicated the existence of polycyclic aromatic compounds with three to four fused benzene rings and/or two to three conjugated systems in unsaturated aliphatic structures (Lumb, 1978; Peuravuori et al., 2002).

The high intensity of peak I in surface samples from RI and RII indicated an important contribution of labile and poorly humified

compounds. This peak was less pronounced for RIII at surface. In the three profiles the relative intensity of peak I decreased at depth, whereas the intensity of peaks II and III increased. This may indicate an increase in the degree of humification at depth, related to an increase in the aromatic character of the FA (Fuentes et al., 2006).

The low fluorescence signals at wavelengths longer than 400 nm highlight the low contribution of highly condensed organic compounds to the FA (Chen et al., 2003). These condensed compounds were typically attributed to lignin or lignin-like compounds (Peuravouri et al., 2002) and frequently found in soil FA (Shin et al., 2001; Senesi et al., 1991).

The humification index obtained from the synchronous spectra (Kalbitz et al., 2000) showed a very good correlation with the Zsolnay index, confirming the increase in humification of FA at depth for the three profiles (Table 3).

3.5. ^{13}C VACP/MAS NMR spectroscopy

The HA ^{13}C NMR spectra are shown in Figure 4. The natural rush presented at surface ($RI_{surface}$) an important contribution of protein-like and methoxyl compounds, as shown by the intensity of the signal at 54 ppm, and the contribution in the alkyl region of the spectrum of branched and short chain compounds (CH_3 signal at 22 ppm) (Baldock et al., 1990). In the polder rush at surface ($RII_{surface}$), the highest content of O-alkyl C was detected (27.5 %), mainly attributable to polysaccharide compounds. The degree of aromaticity of this sample (25.4 %) was lower than in $RI_{surface}$ (31.6 %), and the contribution of the lignin and tannins (160-140 ppm) appeared to be smaller (Figure 4). The surface sample from the lagoon soil ($RIII_{surface}$) was the most different, with a high degree of aromaticity (37.6 %) and an important contribution from long chain alkyl compounds (signal at 30 ppm), probably derived from waxes of vascular plants. Furthermore, two peaks in the aromatic C region (at 148 and 153 ppm) indicated the contribution of lignin and tannin to this spectrum (Kögel-Knabner, 2002). Polysaccharides and proteins were less abundant in this sample than in RI and RII (Table 4).

Table 4. Intensity of ^{13}C (%) in the VACP/MAS ^{13}C NMR spectra, degree of aromaticity (%) and alkyl C/ O-alkyl C ratio.

	Chemical shift range (ppm)							
	245-185	185-160	160-110	110-60	60-45	45-0		
	Carbonyl	Carboxyl/ amide	Aromatic	O-alkyl	N-alkyl/ methoxyl	Alkyl	Ar. (%)	Alkyl/ O-alkyl
Humic Acids								
$RI_{surface}$	4.8	13.2	25.9	20.9	12.1	23	31.6	1.1
RI_{deep}	7	13.7	23.3	18.5	9.9	27.6	29.3	1.5
$RII_{surface}$	4.1	8.4	22.2	27.5	13.5	24.2	25.4	0.9
RII_{deep}	5.7	9	24	23.1	11.8	26.4	28.1	1.1
$RIII_{surface}$	4.9	13.8	30.5	15.9	8.9	26	37.6	1.6
$RIII_{deep}$	3.7	8.2	24.3	23.2	11.4	29.1	27.6	1.3
Fulvic Acids								
$RI_{surface}$	7.2	9.3	19.9	33.9	11.7	18	23.8	0.5
RI_{deep}	6.8	8.2	22.5	29.3	11.4	21.8	26.5	0.7
$RII_{surface}$	5.7	8.2	17.7	40.8	10.6	17.1	20.5	0.4
RII_{deep}	6.2	10	23	22.6	11	27.2	27.5	1.2
$RIII_{surface}$	8.4	7.9	25	20.3	9.6	28.8	29.8	1.4
$RIII_{deep}$	n.c	n.c	n.c	n.c	n.c	n.c	n.c	n.c

n.c = not calculated.

Differences in the HA composition among the deeper layers of the three sites were not as evident as in the surface samples (Fig. 4). The predominant signal in the three spectra was located in the alkyl C region (0-45 ppm), and the degree of aromaticity was relatively low in all spectra (27.6-29.3 %) (Table 4).

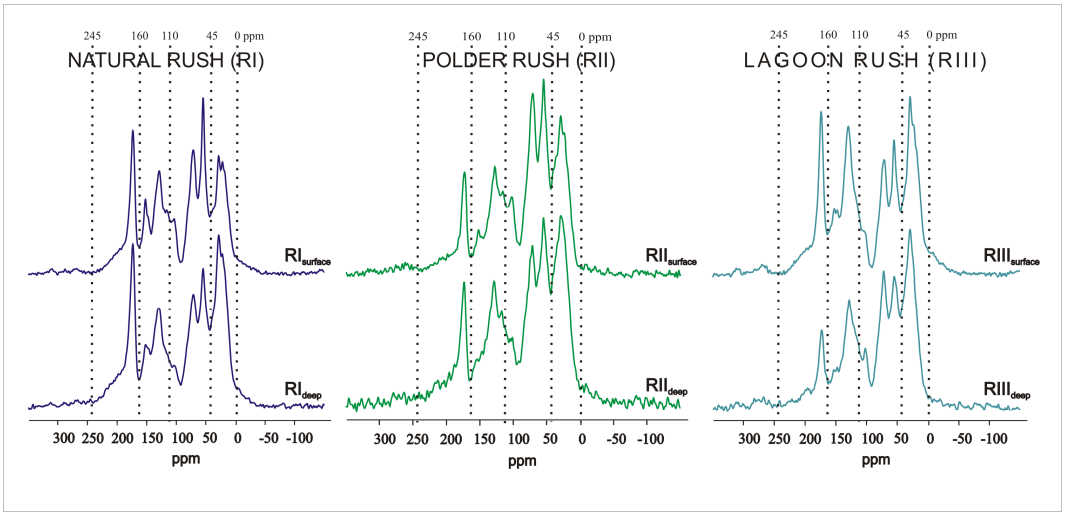

Figure 4. Soil humic acid ^{13}C VACP/MAS NMR spectra.

Variations in the HA composition throughout the profile did not follow a common trend in the three soils. In RI and RII the degree of

aromaticity did not change substantially at depth, although there was some loss of labile and/or poorly humified compounds (45 -110 ppm), and an increase in the alkyl/O-alkyl ratio (Table 4). The greatest differences between the surface and the deep layers were in the lagoon soil (RIII). There was a marked decrease in the degree of aromaticity from 37.6% at the surface, to 27.6 % at depth. The alkyl/O-alkyl ratio also decreased at depth, indicating a higher proportion of labile organic compounds than at the surface. Moreover, the concentration of carboxyl groups was lower (signal at 172 ppm) and the content of carbohydrates increased (peak at 102 ppm). This may suggest a lower degree of humification at depth than at the surface in this soil profile.

As regards the FA (Figure 5), these differed among sites and also with depth in the same profile. At surface, RI and RII were fairly similar, with a predominance of poorly humified compounds, located in the N-alkyl and O-alkyl C regions of the spectra (peaks at 54, 72 and 102 ppm), whereas the aromatic and alkyl compounds were the least abundant (Table 4). Moreover, at depth in both profiles there were increases in the alkyl/O-alkyl ratio and in the degree of aromaticity, which may indicate increases in the humification and decomposition processes (Chabbi and Rumpel, 2004). The lagoon soil (RIII) was again the most different (Figure 5), and though there is no deep layer data for RIII, the surface sample appears to be similar to the deeper samples from the other two profiles (RI_{deep} and RII_{deep}), with a low contribution of labile compounds (45-110 ppm) and high aromatic and alkyl C contents.

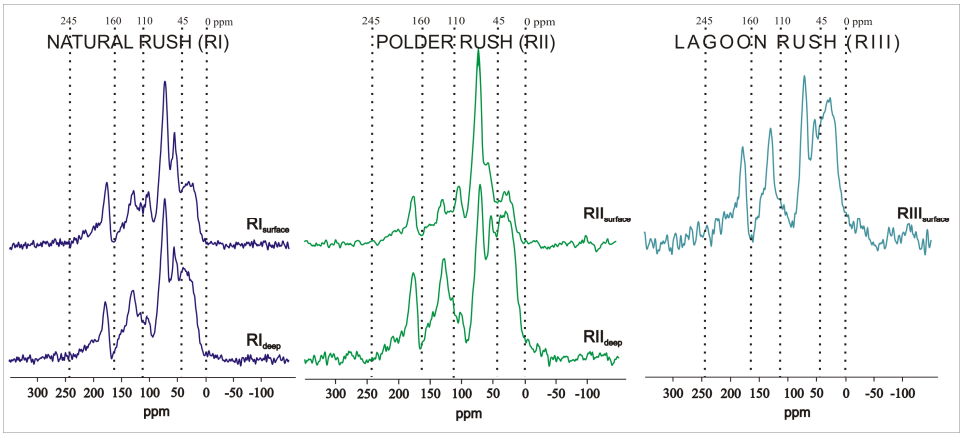

Figure 5. Soil fulvic acid ^{13}C VACP/MAS NMR spectra.

Comparison of the composition of HA and FA revealed evident differences (Table 4). The FA presented a higher content of polysaccharidic compounds (110-60 pm) than their HA counterparts. Such higher contribution of labile compounds in the FA relative to HA has been amply reported (Stevenson, 1994; Preston, 1996; Zech et al., 1997). Furthermore, in all samples the degree of aromaticity is greater for HA than for the corresponding FA. Nevertheless, these values are still lower than those reported for HA from upland soils (Stevenson, 1994; González-Pérez et al., 2004).

4.- Discussion

In the present study, the substrate under *Juncus maritimus* was considered as soil not as sediment because of its capacity to support rooted plants (Soil Survey Staff, 2003). Furthermore, the existence of pedogenetic processes in estuarine substrates has been demonstrated in a recent study (Ferreira et al., 2006). However, the composition of humic and fulvic acids from salt marsh soils under sea rush differed from that generally attributed to humic substances from upland soils (Senesi and Loffredo, 1999). The studied HS were characterized by a low degree of humification and a predominance of simple structural compounds of low aromaticity, mainly polysaccharidic and proteinaceous compounds. These characteristics resemble those attributed to DOM or aquatic humic substances and differ from the typical composition ascribed to soil humic substances (e.g. Chen et al., 2003; Jaffé et al. 2004).

Despite the fact that the vegetation cover was the same (*Juncus maritimus*) in all three sites, the HS compositions differed substantially. The changes in HS composition with depth were also different at each site, and in some cases contrasting trends were observed for humic and fulvic acids.

In the natural rush (RI), the humification process followed the expected increase at depth, for both HA and FA. Although the degree of aromaticity of HA was slightly higher at surface, there was a lower amount of labile compounds, and a higher alkyl/O-alkyl ratio at depth, where the Eh was lower (273 mV). Filip and Alberts (1994b) showed that under

suboxic conditions (Eh 100-300 mV) there was a large increase in the aliphatic C content during humification of salt marsh HA.

The HS in the surface sample from the polder rush soil (RII) presented the lowest degrees of humification. Conditions at this site were suboxic (Eh=108 mV, Table 1) and the lack of oxygen may have reduced the rate of OM decomposition (D'Angelo & Reddy, 1999; Canavan et al., 2006). These suboxic conditions combined with a fine soil texture may have helped protect and stabilize the SOM, therefore leading to the accumulation of relatively fresh OM (see section 3.1). As regards the variations with depth, the HS in the two layers were very similar and, although there was a slight increase in humification of the FA at depth, the trend was not as clear for the HA. There are two possible explanations for the high degree of similarity between the HS at surface and at depth. Firstly, the process of reclamation carried out in this area, which involved mixing of the soil profile, may have homogenized the conditions throughout the profile. Secondly, the low values of the redox conditions of this profile would favour a slow humification process and the preservation of labile compounds at depth.

Finally, HS from the Lagoon rush (RIII) differed most from the other HS, with higher degrees humification and lower contents of labile compounds than those from RI and RII. The low frequency of flooding combined with the sandy texture of the soil resulted in an aerobic environment at the surface, which facilitated rapid decomposition of the fresh organic matter. The most labile compounds, mainly carbohydrates and proteins, were degraded first and this led to a higher degree of humification in these HS comparing with HS from the other sites. Furthermore, the composition of HS at 100 cm depth was particularly interesting as the HS contained more labile compounds and showed a lower degree of humification than in the surface samples. This atypical trend may indicate different past environmental conditions. Thus, the composition of HS in the deeper layer may reflect the existence of other types of vegetation in the past, probably algae, which would lead to a predominance of low aromatic compounds in the HS. Another possibility is fast growth of micro-organisms at depth, which would lead to an

accumulation of labile compounds originating from the microbial biomass (Baldock et al., 1990).

5.- Conclusions

Humic substances in salt marsh soils under sea rush (*Juncus maritimus*) presented a lower degree of humification and a higher content of labile compounds, mainly polysaccharides and proteins, than usually attributed to HS from upland soil. The HS appeared to resemble marine and/or aquatic humic substances.

The plant cover was the same in all studied sites. However, there were important differences in the HS compositions among sites and with depth. HS composition appears to be determined not only by the nature of the original organic material but also by other factors with higher spatial and depth-related variability at local scales, such as physiographical position, flooding regime, soil texture and redox potential.

Further detailed studies about the origin and fate of soil organic matter in these salt marsh environments (e.g. lipid biomarkers and/or organic carbon and nitrogen stable isotopes analyses) should clarify the relationships among HS composition, original organic materials, and the geochemical environment where the humification and decomposition processes take place.

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Capítulo IV. Sustancias húmicas en suelos estuarinos colonizados por *Spartina maritima*

Humic substances in estuarine soils colonized by Spartina maritima

Resumen

En este capítulo se estudian las sustancias húmicas (HS) de dos suelos colonizados por *Spartina maritima* en diferentes posiciones fisiográficas de ambientes estuarinos de la costa noroeste de la Península Ibérica: un punto cercano al canal principal en el Estuario de Villaviciosa y otro punto en la marisma baja de la Ría de Ortigueira. Los ácidos húmicos y fúlvicos se caracterizaron cualitativamente mediante las siguientes técnicas espectroscópicas: infrarrojo por transformada de Fourier, fluorescencia y resonancia magnética nuclear de ^{13}C en estado sólido.

Las SH muestran una predominancia de compuestos poco humificados, con una alta proporción de compuestos alifáticos y un bajo grado de aromaticidad. Además, se encontraron notables diferencias en la composición de las SH de los dos suelos muestreados. En las SH de Villaviciosa, la elevada contribución de compuestos alifáticos no polares y el grado muy bajo de aromaticidad parecen indicar una considerable contribución de compuestos orgánicos de origen marino y/o microbiano. Por el contrario, en las SH de Ortigueira, la mayor proporción de polisacáridos y la presencia de compuestos derivados de la lignina podrían señalar un mayor aporte de compuestos orgánicos derivados de plantas vasculares. La composición isotópica de $\delta^{13}C$ de estos suelos confirma aportes de *Spartina maritima* a la materia orgánica edáfica de Ortigueira, mientras que en Villaviciosa la contribución de esta planta C4 a la materia orgánica edáfica no es tan evidente.

Palabras clave: *Spartina maritima*, sustancias húmicas, estuario, marisma, técnicas espectroscópicas, $\delta^{13}C$.

Abstract

This chapter characterizes humic substances (HS) from two soils colonized by Spartina maritima at different physiographical positions in estuarine environments on the north western coast of the Iberian Peninsula: the Villaviciosa site, a stand close to the main tidal channel, and the Ortigueira site, located in the low salt marsh. Humic and fulvic acids were extracted from the soils and characterized qualitatively by the following spectroscopic techniques: Fourier transform infrared spectroscopy, fluorescence spectroscopy and solid-state ^{13}C nuclear magnetic resonance.

The characterized HS showed a predominance of low humified compounds with a high proportion of aliphatic components and a low degree of aromaticity. The HS composition differed substantially between sites. In the Villaviciosa soil the large amount of nonpolar aliphatic components and the very low degree of aromaticity may indicate a significant contribution of marine organic matter and/or microbial material to the HS. However, in the HS from the Ortigueira soil, the higher proportion of polysaccharides together with the presence of lignin derived compounds may indicate greater inputs of vascular plant material. The $\delta^{13}C$ isotopic composition of the bulk soils highlights the large input of *S.*

maritima debris to the Ortigueira site, whereas in the Villaviciosa site, organic contributions from this C4 vascular plant were not so evident.

The results indicate that in these soils colonized by Spartina maritima, physiographical position has an important effect on the composition of soil HS and, therefore, must be considered in the study of organic matter characteristics in such estuarine environments.

Key words: *Spartina maritima*, humic substances, estuary, salt marshes, spectroscopic techniques, $\delta^{13}C$.

1.- Introduction

The genus *Spartina* is a primary colonist of estuarine mudflats, and develops during the early stages of salt marsh succession. It shows good tolerance to flooded conditions, which allows it to colonize low marsh areas (Maricle and Lee, 2007). Moreover, species of this genus are highly efficient at trapping sediments and protecting against erosion (Neumeier and Ciavola, 2004), which results in elevation of the marsh surface and the subsequent evolution and maturation of these sedimentary systems (Sánchez et al., 2001). The high productivity of these macrophytes is a crucial factor in the ecosystem, as is their capacity to modify the chemistry of sediments and to accumulate metals in the plant tissues and surrounding sediments (Alberts el al., 1990; Otero and Macías 2002, 2003; Reboreda and Caçador, 2007). Furthermore, the presence of these primary colonists enhances fungal and bacterial populations (Nielsen et al., 2001) and also has a positive effect on the salt marsh fauna (Lillebo et al., 1999).

Spartina maritima (Curtis) Fernald mainly occurs on the Atlantic coasts of Europe and Africa. The species is currently in decline, and is being displaced by other introduced species of the same genus, such as *Spartina alterniflora* and *Spartina anglica* (Baumel et al., 2003). Because of the great importance of *Spartina maritima* in European salt marshes, numerous studies have been carried out on the species, and have focused on very different topics such as sediment accretion (Sánchez et al., 2001), productivity (Silva et al., 2005), relationships with nutrients (Otero et al., 2000; Lillebo et al., 2006) and heavy metals (Caçador et al., 2000), and decomposition processes (Castro and Freitas, 2000). However, to our knowledge the organic matter (OM) in soils or sediments colonized by *Spartina maritima* has not been characterized.

Several studies have shown that the low mineralization rates of *S. maritima* detritus cause a large portion of the detritus to remain within the salt marsh (Pozo and Colino, 1992; Lillebo et al., 1999). Thus, *S. maritima* debris is expected to make an important contribution to humic substances

(HS) in salt marsh soils. However, the characteristics of OM in soils and/sediments from estuarine environments are not only determined by the vegetation cover and the chemical composition of the plant material (Wilson et al., 1986), but also by other factors such as the geochemical environment of the soil or sediment (Santín et al., 2008), the contribution of microbial inputs (Tremblay and Benner, 2006) and the root-soil/sediment interactions (Mucha et al., 2005).

Furthermore, in low marshes and tidal flats, where there is a strong tidal influence, allochthonous OM inputs should be also considered. The significance of allochthonous OM inputs, as well as the importance of the exportation of autochthonous OM, greatly depends on the physiographical position that a specific area occupies within the salt marsh or estuary (Taylor and Allanson, 1995; Bouchard and Lefeuvre, 2000; Zhou et al., 2006).

The aim of the present study was to characterize humic acids (HA) and fulvic acids (FA) from soils colonized by *Spartina maritima*. Special attention was given to determining whether or not the position within the estuarine environment results in variations in HS composition, despite the presence of the same vegetation cover. For this purpose, two sites colonized by *Spartina maritima* but in different physiographical positions (a stand near the main tidal channel and a low salt marsh) were selected. Soil HS were extracted and characterized by several spectroscopic techniques, and similarities and differences among the HS are discussed. In order to evaluate the potential sources of soil organic matter (SOM), the $\delta^{13}C$ isotopic compositions of the bulk soils were also determined.

2.- Materials and methods

2.1. Study sites and soil sampling

The selected sites were the Villaviciosa Estuary (43º 31' N 5º 24' W) and the Ria of Ortigueira (43º 41' N 7º 53' W), on the north-western coast of the Iberian Peninsula (Atlantic Ocean). The region is characterized by an oceanic climate, with average temperatures of

between 12 and 14º C and total annual precipitations of between 900 and 1100 mm.

The site locations are shown in Figure 1. Both sites correspond to estuarine environments, which are inundated twice a day by the tide. However, they occupy different physiographical positions: the Villaviciosa site (Fig. 1A) is a single stand of *Spartina maritima* (stem density 645±149 m^{-2}) in a sand bank next to the main channel of the estuary, at 1.0 m above mean sea level; the Ortigueira site (Fig. 1B) is also a homogeneous stand of *S. maritima* (stem density 471±131 m^{-2}), but located at a height of 2.5 m above mean sea level, in the low salt marsh next to the high salt marsh and far away from the tidal channel. This site is a sheltered zone, somewhat protected from tidal and wave energy.

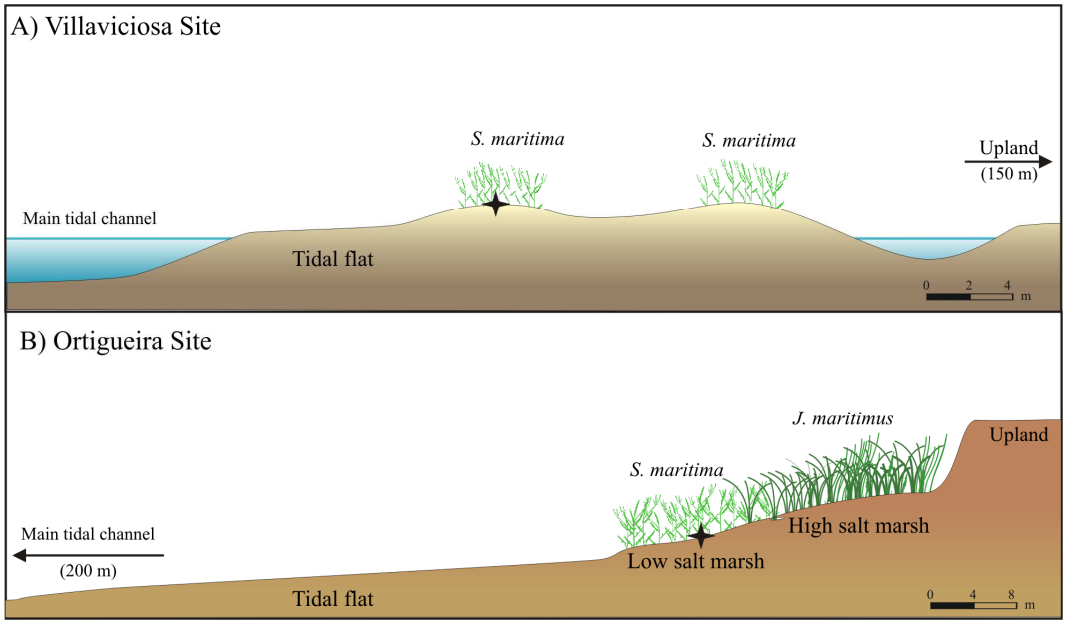

Figure 1. Sampling sites and their physiographical positions within the estuarine environments A) Villaviciosa site; B) Ortigueira site. Stars represent sampling points. Different scales are used in the vertical and horizontal axes.

The sampling was carried out in summer during low tide. At each site, one soil profile was collected in a PVC core (11 cm i.d. and 35 cm in length). The cores were hermetically sealed, stored in an ice box at 4º C, and transported in a vertical position to the laboratory. The cores were cut

into 5-cm sections. Two samples were selected from each site, the first five centimetres of the soil (0-5 cm) and from 20 to 25 cm depth.

2.2. Sample preparation and analysis

The pH and redox potential (Eh) were determined "in situ". The pH was determined with a glass electrode calibrated with pH 4.0 and 7.0 standards, and the Eh with a platinum electrode and after equilibrating the electrode for several minutes. Final Eh readings were corrected by adding the potential (+244mV) of a calomel reference electrode. Soil colour and presence of mottles were determined in fresh samples by use of Munsell soil colour charts.

The concentrations of iron associated with oxyhydroxides (oxyhydroxide-Fe) and with pyrite (pyrite-Fe) were determined in fresh samples. The former was extracted by the citrate-dithionite method and the latter with HNO_3 (Huerta-Díaz and Morse, 1990). Before the extraction of pyrite-Fe, samples were treated with 10 mol L^{-1} HF for 16 h with shaking, to remove iron associated with the silicate. Total iron was extracted by adding 8 ml of a mixture of HNO_3/HF (10:5 v/v) in a 120-ml Teflon bomb containing 0.5 g of dried ground soil, and heating the mixture in an EPTHOS PLUS (Milestone) microwave oven for 20 minutes. The concentration of iron in each extract was determined by flame-atomic-absorption spectrophotometry (Perkin Elmer model 1100B). The concentrations were always expressed in terms of dry weight of soil.

Soil samples were air-dried and sieved (2 mm). Particle size was determined by the pipette method (Gee and Bauder, 1986). Total carbon content (TC) was measured in a LECO CHN-1000 analyzer. To estimate the inorganic carbon content (IC), the organic carbon was eliminated by combustion for 4 h at 450º C (Cambardella et al., 2001) and the TC content was then determined and defined as IC. The total organic carbon (TOC) was estimated as the difference between TC and IC, after prior correction for weight loss.

The stable carbon isotopic compositions ($\delta^{13}C$) of the bulk soils were analyzed in duplicate in an elemental analyzer (EA1108 Carlo Erba)

coupled to a mass spectrometer (MAT253 ThermoFinnigan) (at the Research Support Services, University of A Coruña). Prior to analysis, in order to remove the inorganic carbon, samples were treated with 1 mol L^{-1} HCl and then rinsed in distilled water until complete removal of the acid solution. The $\delta^{13}C$ isotope ratios were expressed relative to the standard reference material (Vienna Pee Dee Belemnite) in parts per thousand (‰):

$$\delta^{13}C\ (‰) = [(R_{sample}/R_{standard})-1] \times 10^{3}$$

where R is the ratio of $^{13}C/^{12}C$.

Humic and fulvic acids were purified according to the International Humic Substances Society methodology (Swift, 1996). Briefly, 100 g of dried sieved soil were extracted with 0.1 mol L^{-1} NaOH, at a soil: solvent ratio of 1:10. After centrifugation, the HA were separated from the supernatant by precipitation with 6 mol L^{-1} HCl until pH 2.0. The precipitated HA were separated by centrifugation, purified by dialysis with a Spectrapor membrane (size exclusion limit, 6000–8000 Da) and freeze-dried. FA were isolated from the acid supernatant in a SUPELITE DAX-8 column (pH 2.0). The DAX-8 column containing sorbed fulvic acid was then rinsed with distilled water and eluted with 0.1 mol L^{-1} NaOH, followed by two column volumes of distilled water (Thurman and Malcolm, 1981). The FA were purified with a Spectrapor dialysis membrane (1000 Da) to remove inorganic salts. The FA in solution were freeze-dried.

Carbon, hydrogen, nitrogen and sulphur contents of HS were determined in a LECO CHNS-932 elemental analyzer. The oxygen content was measured in a LECO-VTF-900 graphite furnace. Ash content was calculated by difference (100% - %CHNSO).

2.3. Spectroscopic characterization of humic substances

FTIR Spectroscopy

Spectra were recorded in a Fourier-Transformed Infrared Perkin Elmer, Paragon 1000 PC spectrophotometer. The KBr pellets were obtained by pressing the mixture (1 mg HS: 100 mg KBr, spectrometry grade) at 10 000 kg cm^{-2} for 2 minutes (Stevenson, 1994). Spectra were

acquired at 4 cm^{-1} resolution, and 64 scans were averaged to reduce noise.

Fluorescence Spectroscopy

Fluorescence experiments were carried out with HS dissolved in aqueous solutions of $NaHCO_3$ (0.05 mol L^{-1}), at a concentration of 20 mg L^{-1} and ~pH8. These conditions were evaluated as the most suitable to avoid inner filter effects (Milori et al., 2002). Spectra were recorded in a Perkin Elmer LS 50B luminescence spectrometer with a scanning speed of 200 nm min^{-1}. The emission and the excitation slits were set at 10 nm for HA and reduced to 7 nm for FA due to the higher fluorescence intensity of FA. For each measurement, the corresponding spectrum of the blank solution was subtracted from those of the samples.

Emission spectra were measured with an excitation wavelength of 240 nm over a range of 300 to 600 nm. A humification index was estimated from emission spectra, according to Zsolnay et al. (1999). For FA, synchronous-scan spectra were also recorded, over a range of 200 to 600 nm, by simultaneous scanning of both the excitation and emission wavelengths while keeping a constant wavelength difference ($\Delta\lambda$ = 18 nm). This difference has been observed to provide the best optimized resolution for HS spectra (Peuravuori et al., 2002).

^{13}C VACP/MAS NMR Spectroscopy

Solid-state ^{13}C NMR spectra were acquired with a Bruker DSX 200 spectrometer at a ^{13}C resonance frequency of 50.32 MHz. The samples were confined in a zirconium oxide rotor with an external diameter of 7 mm. The variable amplitude cross polarization magic angle spinning technique (VAC/MAS) was applied with a contact time of 1 ms, a spinning speed of 6.8 kHz and a pulse delay of 1 s. The spectra were subdivided into different chemical shift regions according to Knicker and Lüdemann (1995): alkyl C (0–45 ppm), N-alkyl/methoxyl C (45-60 ppm), O-alkyl C (60–110 ppm), aromatic C (110–160 ppm), carboxyl/amide C (160-185 ppm) and carbonyl C (185–245 ppm). The relative ^{13}C intensity distribution

was determined by integrating the signal intensity in different chemical shift regions with an integration routine supplied by the instrument software. Corrections for the intensity distribution of spinning side bands were made (Knicker et al., 2005). Moreover, the degree of aromaticity (%) (aromatic-C * 100 / (alkyl-C + N-alkyl/methoxyl C + O-alkyl-C + aromatic-C)) (Hatcher et al., 1981) was calculated.

3.- Results

3.1. General characterization of soils

Some descriptors of the studied soils are shown in Table 1. The pH of both Villaviciosa and Ortigueira soils was approximately neutral (6.9-7.6), the organic carbon content was low and decreasesd with depth (1.0-3.2%), and the inorganic carbon content was very low (0.1-0.6%). At the surface, the Eh potential was similar in both sites, and indicated suboxic conditions (Eh: 100-300 mV). However, the presence of pyrite-Fe and the abundance of black mottles (iron monosulphides) at the surface of both soils (Table 1) also indicate the existence of discrete anoxic microenvironments where sulphate reduction occurs (Otero and Macías, 2002, 2003). The most important difference observed between sites was the soil texture, sandier in Villaciosa, both at surface and at depth (Table 1). Furthermore, in the Ortigueira site there was a decrease in the Eh and in the concentration of oxyhydroxide-Fe with depth, and an increase in the concentration of pyrite-Fe, whereas in the Villaviciosa site the redox potential increased and the pyrite-Fe decreased with depth (Table 1). The pattern observed in Ortigueira soil is consistent with those reported for similar low marshes colonized by *Spartina maritima*, with suboxic conditions at the surface and anoxic conditions below 15 cm depth (Otero and Macías, 2002, 2003). However, the characteristics of the Villaviciosa soil indicate a substrate in which the plants have little or no effects on the soil at depths below 15 cm. Additionally, in the Villaviciosa site, washing of the organic matter by tidal action is facilitated by the sandy substrate, which may limit the activity of sulphate reducing bacteria. The lack of microbial activity is also confirmed by the high Eh value at depth (487 mV).

The $\delta^{13}C$ values (‰) for the bulk soils are reported in Table 1. The isotopic signature of OM depends on the carbon sources and the biosynthetic pathways that the different primary producers use for carbon fixation. Therefore, the $\delta^{13}C$ values (‰) can be used to distinguish among different sources of OM in soils and sediments such as C3 and C4 vascular plants and algae (Goñi et al., 1997). *Spartina maritima* is a vascular plant with a C4 metabolism, and thus its organic composition is enriched in $\delta^{13}C$. Although both studied soils are colonized by *S. maritima*, their bulk soils differ greatly in the $\delta^{13}C$ isotopic signature. In the Villaviciosa soil the SOM is much more depleted in $\delta^{13}C$ (-24.0 at 0-5 cm depth and -23.8‰ at 20-25 cm depth) than in the Ortigueira soil (-18.4 at 0-5 cm depth and -18.9‰ at 20-25 cm depth).

Table 1. Description of soil samples and parameters analyzed.

	Villaviciosa Site		Ortigueira Site	
	0-5 cm	20-25 cm	0-5 cm	20-25 cm
Tidal flood frequency	Twice a day		Twice a day	
Soil colour	dark grey	dark grayish brown	grey	grey
Mottles	black	reddish	black and reddish	none
pH	7.6	7.6	6.9	7.2
Eh (mV)	218	487	177	125
% TC	3.8	1.4	3.0	2.0
% TOC	3.2	1.0	2.9	1.9
% IC	0.6	0.4	0.1	0.1
% Fe	1.9	1.4	3.4	3.3
Oxyhydroxides-Fe (µmol/g)	219.9	145.2	136.0	60.7
Pyrite-Fe (µmol/g)	3.9	0.3	47.3	163.0
% Sand	37.2	80.9	2.9	8.2
% Silt	42.9	3.2	62.3	60.5
% Clay	19.9	15.8	34.8	31.3
$\delta^{13}C$ (‰)	-24.0	-23.8	-18.5	-18.9

3.2. Characterization of soil humic substances

3.2.1. Elemental Composition

During the HS purification procedure neither humic nor fulvic acids were obtained from the Villaviciosa soil at depth (20-25 cm), probably

because of its very low organic carbon content (1.0%). Moreover, sufficient FA was not able to be extracted from Ortigueira soil at depth. This may be because FA are soluble throughout the range of pH and the soil in these environments is subjected to constant washing by the seawater during tidal flooding. Decreases in the total amount of HA and FA with depth have been reported in previous studies (Kappler et al., 2001; Calace et al., 2006; Tremblay and Gagné, 2007).

The elemental composition and atomic ratios of the studied HS are shown in Table 2. The C and N contents were higher in HA than in FA, but the H and O contents are lower. The S content was high in all the samples (1.3-3.2%) when compared with typical values attributed to HA (0.1-1.5%) and FA (0.1-3.6%) (Senesi and Loffredo, 1999). Furthermore, higher S contents were found in the samples with lower Eh values (Table 1). This may be attributed to enrichment of sulphur in the HS via sulphur reducing reactions that occur under anoxic conditions (Brüchert and Pratt, 1996).

The atomic C/N ratios of the studied HS (Table 2) were lower than those reported for *Spartina alterniflora* salt marshes (Filip and Alberts, 1994, Wang et al., 2003; See and Bronk, 2005), but similar to those obtained for sedimentary and marine HS (Calace et al., 2006; Liu et al., 2006). Furthermore, lower C/N ratios were obtained for the Villaviciosa HS (9.0-9.6) than for the Ortigueira HS (11.7-14.4). This may indicate a higher marine and/or microbial contribution to the Villaviciosa HS since these materials contain more nitrogen than typical vascular plant compounds, such as lignin or tannins, which have very low N contents.

Moreover, the high O/C ratios (0.5-0.7) indicate a large content of O-containing groups, and the high H/C ratios (1.3-1.8) a predominance of aliphatic compounds in these estuarine HS (Senesi et al., 2003).

No marked differences in the elemental composition of Ortigueira HA between surface and depth were found, only the already mentioned enrichment of S at depth and a slight increase in the C/N ratio (Table 2).

Table 2. Elemental composition (ash-free, expressed as %), atomic ratios and percentage of ash of humic and fulvic acids.

	N	C	H	S	O	C/N	H/C	O/C	% Ash
Humic Acids									
Villaviciosa 0-5 cm	6.4	49.0	5.8	1.3	37.4	9.0	1.4	0.6	7.7
Ortigueira 0-5 cm	5.2	52.5	5.9	1.7	34.8	11.7	1.3	0.5	6.2
Ortigueira 20-25 cm	4.9	51.3	5.4	3.2	35.2	12.3	1.3	0.5	3.9
Fulvic Acids									
Villaviciosa 0-5 cm	5.3	43.8	6.6	1.4	42.9	9.6	1.8	0.7	13.9
Ortigueira 0-5 cm	3.8	46.9	6.5	1.8	41.0	14.4	1.7	0.7	11.8

3.2.2. FTIR Spectroscopy

The FTIR spectra of surface (0-5 cm) HA and FA are shown in Figure 2. Identification of the main absorption bands was based on data published by Stevenson (1994) and Silverstein et al., (2005). HA spectra are very similar to those recorded for HS released from dead *Spartina alterniflora* plants (Filip and Alberts, 1988) and to those obtained from HA from surface sediments in a salt marsh of the Ria de Aveiro, Portugal (Mendonça et al., 2004).

Although the bands in both HA and FA spectra are very similar, there are differences in the relative intensities (Fig. 2). The intensity of the first band, centred at 3400 cm^{-1}, attributed to O-H and N-H stretching is higher in the FA, as are the intensities of the peaks at 1650 cm^{-1} and 1390 cm^{-1}. These peaks are attributed to the C-O asymmetric and symmetric stretching of the anion carboxylate (COO^-), respectively. It has been shown that the band assigned to the C=O stretching of the COOH may shift from 1710-1720 cm^{-1} to 1650 cm^{-1} for many organic acids due to resonance effects (Silverstein et al., 2005). Taking this into account the band at 1650 cm^{-1} can be attributed both to vibrations of the carboxylic groups and also to the asymmetric stretching of COO^- groups. The high intensity of this band together with the high intensity of band at 1390 cm^{-1} and the peak at 1230 cm^{-1}confirms the high content of carboxylic groups in FA.

In the HA spectra, the shoulder at 1715 cm^{-1} (C=O stretching of COOH) in conjunction with the peak at 1230 cm^{-1} (C-O stretching and OH deformation of COOH) indicate the presence of carboxyl groups. The peak assigned to asymmetric stretching of methylene groups, at 2930 cm^{-1}, is

more evident in the HA spectra than in the FA spectra. Furthermore, the amide II band (at 1590-1517 cm^{-1}) and the amide I band (at 1660-16630 cm^{-1}) suggest the presence of protein-like compounds in the HA. In the FA, both amide bands are probably obscured by the band at 1650 cm^{-1}. The peak at 1080-1030 cm^{-1}, ascribed to the C-O stretching and Si-O vibrations, is more intense in the Villaviciosa HA than in the corresponding FA spectrum, although it more intense in Ortigueira FA than in the HA spectrum.

As regards differences between sites, Villaviciosa HA display greater intensities at 1640 and 1530 cm^{-1} than the Ortigueira HA, which may be related to higher contents of protein-like compounds. Also the peak at 1080-1030 cm^{-1} is higher in the Villaviciosa HA. As regards the FA, the spectra from the two sites (Fig. 2) were very similar, with a higher C-O and/or Si-O signal in Ortigueira (band at 1080-1030 cm^{-1}).

Impurities in the HS are expected to be observed in the band at 1080-1030 cm^{-1}, which is partially assigned to the stretching vibrations of the Si-O group. However, no clear relationships were observed between the intensity of this signal (Fig. 2) and the percentage of ash in each of the studied HS (Table 2).

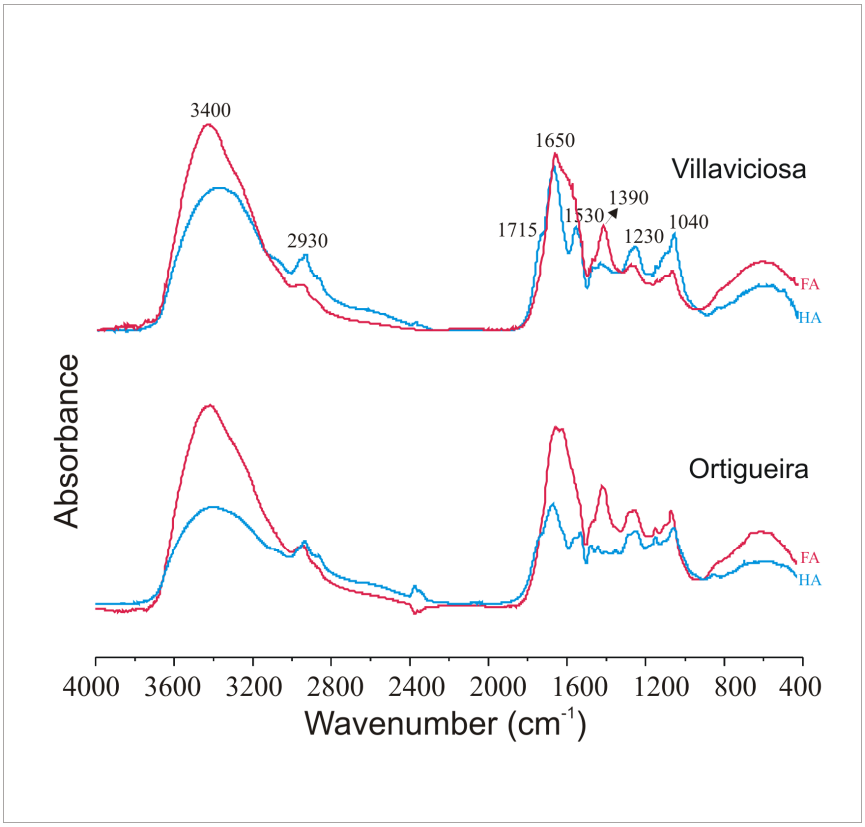

Figure 2. FTIR spectra of surface (0-5 cm) soil humic (blue line) and fulvic (red line) acids from Villaviciosa and Ortigueria sites.

3.2.3. Fluorescence Spectroscopy

The emission spectra of the surface HA (0-5 cm) show a main broad band, centred at 420 nm, and also another small band at shorter wavelengths (Fig. 3A). In general, higher emission intensities at shorter wavelengths indicate a predominance of simple structural components with a low degree of aromaticity and low levels of conjugated chromophores (Senesi et al., 1991). Thus, Villaviciosa HA are less humified than Ortigueira HA because of the higher intensity of the first band and the proportional lower intensity of the main band (Fig. 3A). The humification index calculated from the emission spectra (Zsolnay et al., 1999) confirms a higher degree of humification in Ortigueira HA (0.22) than in Villaviciosa HA (0.17) at the surface (0-5 cm); moreover, the degree of humification increases at depth in the Ortigueira HA (0.38 at 20-25 cm depth; data not shown). As regards the emission spectra of the

surface FA, the two spectra are very similar, with only a single main band centred at 420 nm (Fig. 3B). The humification index is higher for Villaviciosa FA (0.46) than for Ortigueira FA (0.33).

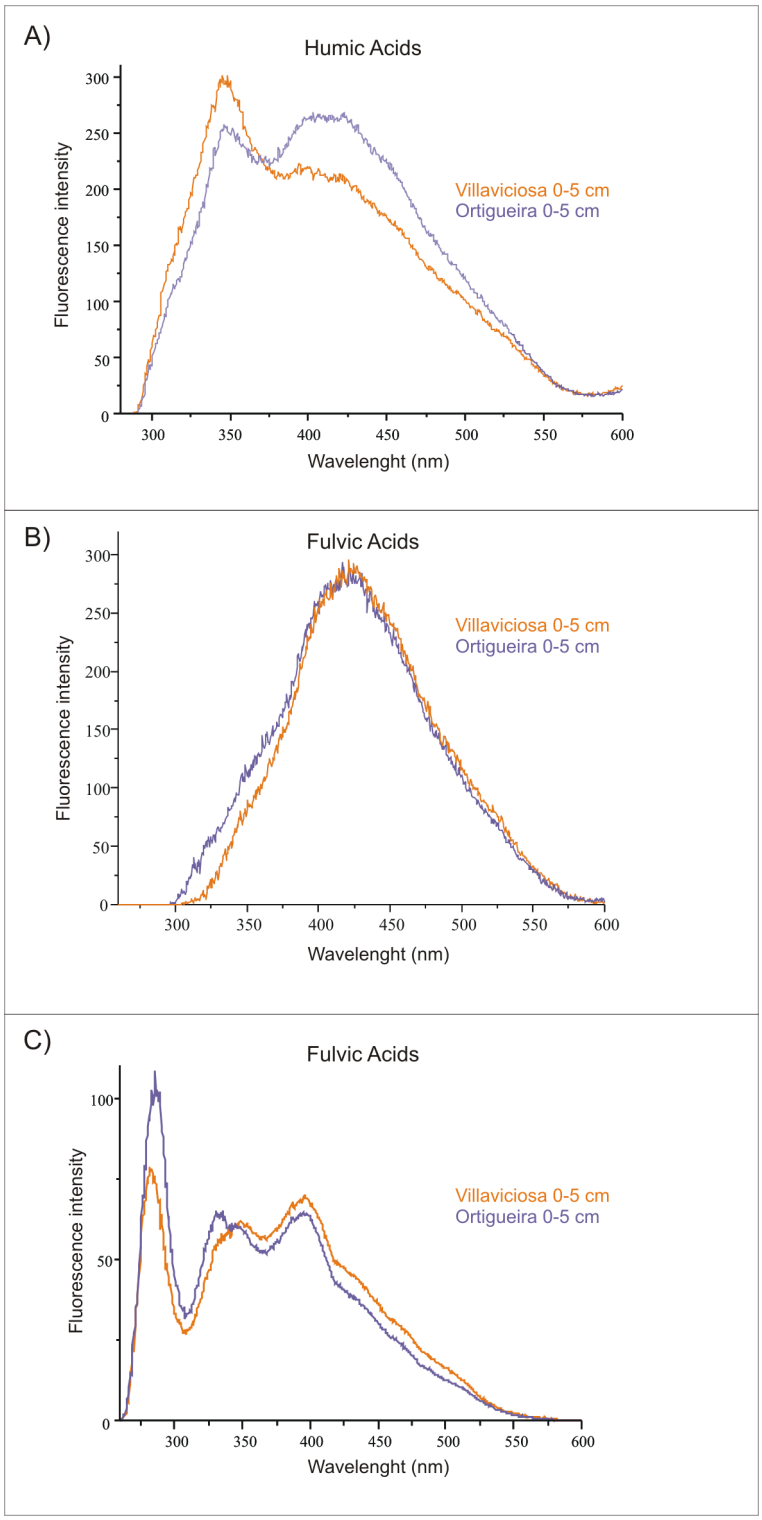

Figure 3. Fluorescence spectra (A) HA Emission spectra (λ_{exc} = 240 nm); (B) FA Emission spectra (λ_{exc} = 240 nm); (C) FA Synchronous-scan spectra ($\Delta\lambda$ = 18 nm).

The synchronous-scan spectra of the surface (0-5 cm) FA are shown in Fig. 3C. This synchronous-scan mode reduces the overlapping interferences, allowing better identification of each fluorescent component (Peuravuori et al., 2002). Thus, even though both spectra are quite similar, each with three peaks, more differences were observed between them (Fig. 3C) than between the corresponding emission spectra (Fig. 3B). The first peak (280 nm), more intense in Ortigueira FA, is attributable to proteinaceous materials and/or mono-aromatic compounds (Ferrari and Mingazzini, 1995). The other two, located at around 350 and 400 nm, respectively, are related to the existence of polycyclic aromatic hydrocarbons with two to four fused benzene rings and/or two to three conjugated systems in unsaturated aliphatic structures (Lumb, 1978). The high intensity of the first peak in both spectra highlights the predominance of low humified material, mainly proteinaceous compounds and/or phenolic units. In the Villaviciosa FA, with a lower C/N ratio (9.6), the first peak in the synchronous-scan spectra may be mainly due to proteinaceous compounds, whereas in the Ortigueira FA, with a higher C/N ratio (14.4), phenolic moieties resulting from lignin degradation may also be important (Ferrari and Mingazzini, 1995). Furthermore, the low intensities at long wavelengths (> 440 nm) in both spectra indicate a low contribution of humified and condensed compounds to these estuarine FA (Miano and Senesi, 1992).

3.2.4. ^{13}C VACP/MAS NMR Spectroscopy

The ^{13}C distribution of the studied HS, represented by the percentage of total signal intensity in each region of the spectrum, is summarized in Table 3. The spectra of the surface HA and FA are shown in Figure 4.

Comparing surface HA (Fig. 4.A), the Villaviciosa HA display a lower aromatic C content and higher proportion of alkyl C (0-45 ppm) than Ortigueira HA. In the Villaviciosa HA, in the alkyl C region, a peak at 22 ppm supports the existence of short-chain alkyl structures attributable to methyl groups probably present in lipids, hemicelluloses (Kögel-Knabner,

2002) or peptide structures (Knicker, 2000). Furthermore, this spectrum presents the highest signal at 174 ppm (Fig. 4). This sharp peak at the carboxyl region is ascribed mainly to carboxyl and/or amide groups. Taking into account that Villaviciosa HA also shows the highest N content (Table 2), it is possible an important amide contribution to this peak. On the other hand, in the Ortigueira HA, the peak at 174 ppm is of lower intensity than in the Villaviciosa HA, and also lower in intensity compared with the peak at 55 ppm ascribed to N-alkyl and methoxyl C (Fig. 4.A). This suggests that in Ortigueira HA a high proportion of the signal at 55 ppm is due to methoxyl groups from lignin derived compounds, since a major contribution of N-alkyl (proteinaceous material) would also be reflected by a higher intensity at 174 ppm. Moreover, the presence of plant derived materials in the Ortigueira HA is also indicated by the higher degree of aromaticity, and the presence of tannins, lignin, and/or lignin derived compounds is corroborated by the above-mentioned peak at 55 ppm and also by three peaks in the aromatic C region at 115, 146 and 153 ppm (Kögel-Knabner, 2002). The peak at 115 ppm is absent from the Villaviciosa HA spectrum and the other peaks, at 146 and 153 ppm, are poorly defined (Fig. 4A). This suggests a lack or a low amount of lignin and tannins in the Villaviciosa HA. Hence, the Villaviciosa HA display a very similar qualitative composition to HS derived from microbial or algae material, whereas the Ortigueira HA resemble humic material from vascular plants (Kögel-Knaber, 2002).

Variations in the composition of the Ortigueira HA with depth were observed (Table 3). The most intense changes were an increase on the nonpolar aliphatic C contribution and a decrease in O-alkyl C compounds. Furthermore, the increase in the carboxyl/amide signal at depth and the decrease of intensity in the N-alkyl/methoxyl peak may be attributed to the degradation of lignin-derived compounds (Kögel-Knaber, 2002).

Surface FA spectra were more similar to each other than the surface HA spectra (Fig. 4B). In both, the signal at 70 ppm was the most intense, which indicates an important contribution of polysaccharides. Furthermore, the alkyl C region (0-45 ppm) was dominated by a signal

peaking at 28-30 ppm, typical of the internal methylene C in long alkyl chains (Preston, 1996). As observed for the HA, the degree of aromaticity was higher in the Ortigueira FA than in the Villaviciosa FA. Interestingly, this may be considered as inconsistent with the lower degree humification estimated from the fluorescence emission spectra in Villaviciosa FA compared with Ortigueira FA (see section 3.2.3). However, this can be clarified by considering that FA are mainly constituted by low molecular weight compounds. Therefore, it can be suggested that in the Ortigueira FA, an important part of the aromatic signal in the NMR spectra is attributable to lignin-derived phenolic moieties, which, in the fluorescence spectra would contribute to the signal at lower wavelengths and therefore would not increase the degree of humification estimated by fluorescence (Zsolnay et al., 1999). This is unlikely to apply to the HA, as the corresponding degrees of aromaticity and humification are related to more conjugated systems and to associations of larger organic compounds.

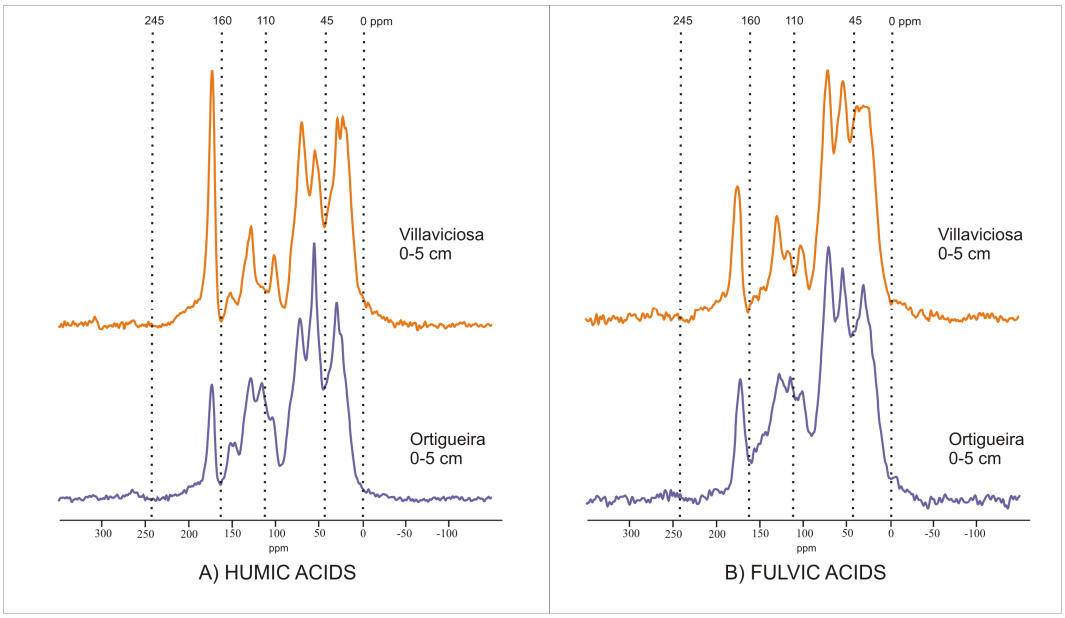

Figure 4. ^{13}C VACP/MAS NMR spectra of (A) humic acids and (B) fulvic acids from soils colonized by *Spartina maritima*.

Table 3. ^{13}C intensity distribution (%) in the VACP/MAS ^{13}C NMR spectra and degree of aromaticity (Ar. %).

	Chemical shift range (ppm)						
	245-185	185-160	160-110	110-60	60-45	45-0	
	Carbonyl	Carboxyl/ Amide	Aromatic	O-Alkyl	N-Alkyl/ Methoxyl	Alkyl	Ar. (%)
Humic Acids							
Villaviciosa 0-5 cm	2.9	14.3	14.9	23.8	12.0	32.1	18.0
Ortigueira 0-5 cm	1.8	7.3	23.2	26.9	15.0	25.8	25.6
Ortigueira 20-25 cm	4.6	12.1	23.7	16.3	11.9	31.4	28.4
Fulvic Acids							
Villaviciosa 0-5 cm	2.9	8.3	16.0	26.2	14.5	32.0	18.0
Ortigueira 0-5 cm	1.7	8.1	22.6	29.0	12.6	25.9	25.1

4.- Discussion

The HS composition in the soils under study differed substantially, even though both soils are colonized by *Spartina maritima*. Interestingly, the results obtained with all the spectroscopic techniques used in this study revealed greater differences between the characterized HA than between their FA counterparts.

The differences detected in HS composition between sites with the same vegetation cover may be attributed to differences in the geochemical environment. Filip and Alberts (1994) found that the diagenetic transformations of HS from *S. alterniflora* debris are determined by the oxygen availability, with an increase in aromatic compounds under aerobic conditions and greater aliphatic enrichment and a decrease in the C/N ratio under semi-aerobic conditions. In a previous study of HS from high salt marsh soils under *Juncus maritimus*, Santín et al., (2008) reported an increase in the degree of humification of HS related to higher Eh values and predominance of sand in the mineral fraction of the soil. Tremblay and Gagné (2007) also shown that coarser textures promote degradation of SOM. From these previous studies, an increase in the degree of humification of HS would be expected under more oxic conditions and sandier textures. However, in the present study the degree of humification followed the opposite trend. HS from Ortigueira soil, which has finer texture and a lower Eh potential (see section 3.1), displayed a higher degree of aromaticity and a lower proportion of poorly humified components. Other environmental factors must therefore be analyzed, to

clarify the causes of these differences. Both sites are situated in the low marsh and tidal flat and subject to tidal influence, therefore, possible allochthonous OM inputs should also be considered.

Some of the research on *S. alterniflora* has demonstrated that plant tissues make an important contribution to salt marsh HS (e.g.: Filip and Alberts, 1988; Alberts and Filip, 1989), while others have also found additional OM sources such as upland inputs or algal remains (Alberts et al., 1988; Goñi and Thomas, 2000). In the present study, the studied HS share several characteristics such as a low C/N ratio, low degree of aromaticity and a high proportion of carbohydrates and proteins. We suggest that these common features may be related to inputs of non-terrestrial organic materials to these HS, especially in the case of Villaviciosa HS. The significant contribution of sources other than vascular plants is supported by the predominance of low humified components and by the greater contribution of aliphatic compounds rather than aromatic structures. Moreover, although lignin-derived structures are reported to be important constituents of the SOM in salt marshes (Klap et al., 2000; Filip et al., 1991; Craft et al., 1988), these compounds are not as abundant as expected in the studied HS, especially in the Villaviciosa site.

In order to elucidate the main sources of OM in these two sites, the $\delta^{13}C$ isotopic signature of the bulk soils was plotted against the atomic N/C ratio of the HS (Fig. 5). The compositional ranges of some potential sources of SOM were also included in the graph. The values obtained for the two soils differ from those reported for potential sources. This was expected because SOM is a mixture of different components and also because the original organic matter is altered by the decomposition and humification processes that take place in the soil. Moreover, the $\delta^{13}C$ isotopic signatures of both soils are very different (Fig. 5); therefore, they presumably differ in the main sources of organic inputs to the SOM.

As regards the Ortigueira soil, the isotopic signature of the SOM indicates a predominance of autochthonous inputs from *Spartina*. Although it is slightly more depleted in ^{13}C than *S. maritima* tissues ($\delta^{13}C$ = 13.5‰, Vinagre et al., 2008), the values are consistent with those found in

salt marsh soils colonized by *Spartina* (Alberts et al., 1988; Fogel et al., 1989; Bull et al., 1999; Goñi and Thomas, 2000). One plausible explanation for this depletion is the contribution of microbial inputs, since they comprise a major fraction of the preserved OM from *Spartina* detritus (Tremblay and Benner, 2006). Furthermore, the observed depletion may also be partially attributed to changes in the isotopic composition of the OM derived from plants during the decomposition process in the soil. This is attributed to the preferential decay of *Spartina* polysaccharides and proteins and the preservation of lipids and lignin, which lead to isotopically depleted SOM (Benner et al., 1987).

As regards variations with depth in Ortigueira, the isotopic signature of the SOM is similar at the surface (-18.5‰) and at depth (-18.9‰), which indicates similar sources for the SOM. Thus, the observed differences in HA characteristics with depth may be attributed to the humification and decomposition processes that occur at different depth. The decrease in proteins and polysaccharides in the HA at depth is consistent with the preferential degradation of non-lignin components during the humification process of *S. alterniflora* under subaerial conditions referred to by Filip et al. (1991). Moreover, the enrichment of nonpolar alkyl compounds is consistent with results obtained by Filip and Alberts (1994) under semi-anaerobic conditions and also with those reported by See and Bronk (2005). On the other hand, Filip and Alberts (1994) and See and Bronk (2005) also observed a decrease in the C/N of the HS during humification, which may be related to the N-immobilization mechanism mediated by bacteria (Tremblay and Benner, 2006). However, no decrease in the C/N with depth was detected in the present study (see section 3.2.1).

The SOM in the Villaviciosa soil had a lower $\delta^{13}C$ (23.8-24.0‰) and HS with a higher atomic N/C ratio than the SOM in the Ortigueira soil (Fig. 5). In this case, the $\delta^{13}C$ values are too low to be explained by the selective preservation of isotopically depleted compounds during the decomposition process (Goñi and Thomas, 2000), since the $\delta^{13}C$ values of *Spartina* lignin and lipids are only 5‰ units lower than the bulk TOC (approx. -13‰.). Consequently, the most likely explanation is that in the

Villaviciosa site, a substantial part of the SOM is derived from other sources with a more depleted carbon isotope signature than *Spartina* and richer in nitrogen, such as phytoplankton or benthic algae (Middelburg et al., 1997; Gebrehiwet et al., 2008). Contributions from terrestrial C3 plants may also decrease the $\delta^{13}C$ ‰; however a significant C3 plant input is unlikely since the observed $\delta^{13}C$ values are heavier than those usually reported for C3 plants (average -26‰). Additionally, the present results from the Villaviciosa site can be compared with those previously reported by the same authors from two salt marshes covered by *Juncus maritimus* in the same estuary (Santín et al., 2008). Differences are noteworthy, with a much lower degree of aromatic, larger nonpolar aliphatic contributions, lower C/N ratio and lower humification in the HS from the present study. This appears to confirm that no remarkable inputs from marsh plants are reflected in the HS composition from the Villaviciosa soil. Thus, we suggest that in the Villaviciosa site, the soil OM is mainly derived from non-terrestrial estuarine and/or marine sources such as benthic macroalgae (Currin et al., 1995), edaphic algae (Sullivan and Moncreiff, 1990), seston (Sherr, 1982) or plankton (Peterson et al., 1985; Sullivan and Moncreiff, 1990; Malet et al., 2008, Gebrehiwet et al., 2008). Importantly, as can be deduced from Fig. 5, a ubiquitous microbial contribution to OM should not be discounted (Tremblay and Benner, 2006).

Therefore, in Villaviciosa, the lack of any evidence showing that the OM is derived from vascular plants may suggest an important export of *S. maritima* primary production. Furthermore, a significant allochthonous contribution to the soil OM, mainly from aquatic systems, is reflected in both the HS composition and the $\delta^{13}C$ values of the bulk soil. The physiographical position of the Villaviciosa site, a highly exposed area near the main tidal channel may favour such interchange processes.

On the other hand, the Ortigueira salt marsh soil contained more humified HS and evidence of lignin derived compounds. Moreover, the isotopic composition indicates an important autochthonous contribution of *Spartina* to the Ortigueira SOM. It is therefore possible that export of OM

out of this area is more limited, because it is largely sheltered from the effects of tidal movements. This would facilitate accumulation of autochthonous OM from *S. maritima* in the soil.

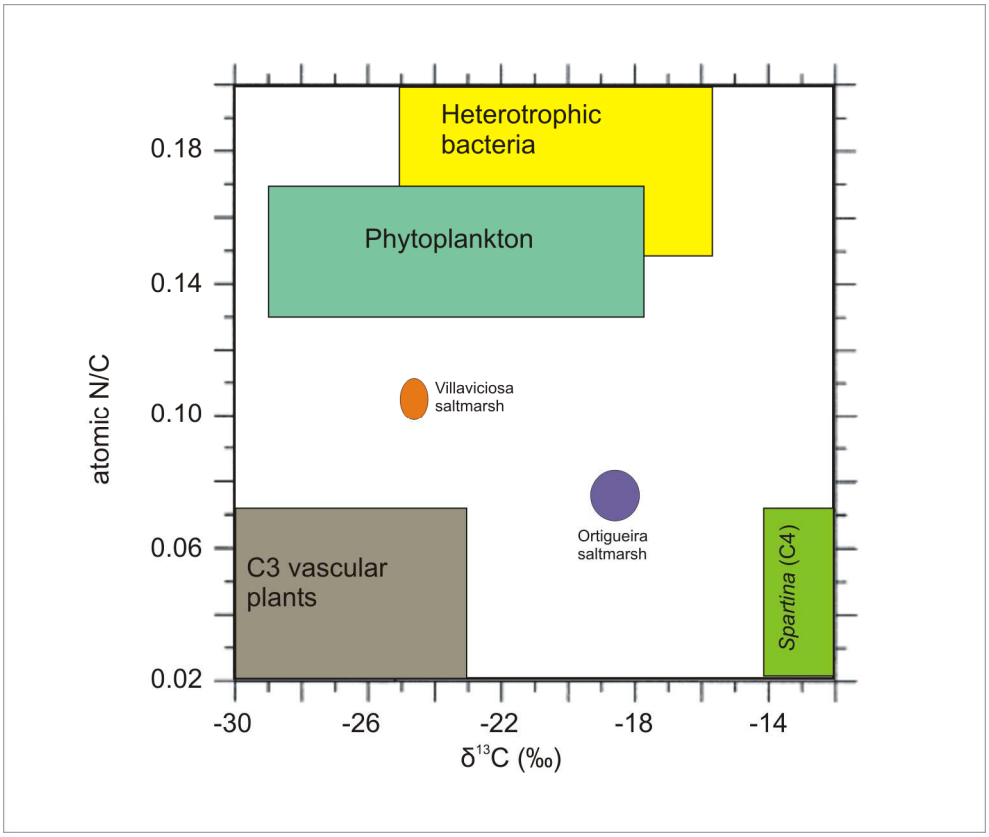

Figure 5. Stable carbon isotopic compositions ($\delta^{13}C$) plotted against atomic nitrogen/carbon ratios for some potential sources of SOM in the studied salt marsh soils (graph modified from Goñi et al., 2003). The orange circle represents the atomic N/C ratios of humic substances from Villaviciosa site plotted against the $\delta^{13}C$ values recorded for this bulk soil. The violet circle represents the same parameters for the Ortigueira site.

5.- Conclusions

The spectroscopic characterization of humic and fulvic acids from estuarine soils colonized by *Spartina maritima* shows a predominance of aliphatic compounds, a low contribution of aromatic structures and a low degree of humification. Nevertheless, although the vegetation cover was the same there were important differences in the HS composition at the two sites. The Villaviciosa soil appears to contain more marine organic material as a consequence of its greater exposure to the sea. On the other

hand, the SOM in the Ortigueira site contains large amounts of *S. maritima* debris, which is reflected both in the isotopic signature of the SOM and in the composition of the humic substances. Finally, coastal environments are complex and dynamic sedimentary systems where the SOM can be derived from autochthonous as well as allocthonous sources, and the systems can also act both as sinks for OM, and sources of OM for adjacent ecosystems. Therefore, further studies regarding the export and import of OM should be carried out for in order to improve our understanding of OM dynamics in estuarine environments.

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Capítulo V. Efecto de los procesos de reclamado y regeneración en la materia orgánica de suelos y sedimentos estuarinos

Effects of reclamation and regeneration processes on organic matter from estuarine soils and sediments

Resumen

En este capítulo se investigan los cambios en la materia orgánica (MO) relacionados con los procesos de reclamado y regeneración de suelos y sedimentos en ambientes estuarinos. Para ello se seleccionaron tres zonas adyacentes en la marisma alta del Estuario de Urdaibai (NW de España): una zona reclamada, una zona natural y una zona regenerada. La MO presente en los perfiles seleccionados se caracterizó mediante resonancia magnética nuclear de ^{13}C en estado sólido (NMR) y pirólisis analítica (Py-GC/MS).

La marisma reclamada presenta características edáficas muy diferentes en todo su perfil a las de la marisma natural, indicando una importante alteración de los parámetros geoquímicos y de la composición de la MO por el proceso de reclamado. En la marisma reclamada, con condiciones óxicas y falta de influencia mareal, la transformación de marisma a pastizal conllevó un cambio en los aportes de MO, ahora más rica en carbohidratos y con menor proporción de compuestos aromáticos que la MO de suelos de marisma. Por otra parte, las características edáficas de la marisma regenerada son similares a la de la marisma natural y también la vegetación es la misma. Sin embargo, la marisma natural acumula en superficie el doble de carbono orgánico que la marisma reclamada. Además, los resultados de NMR indican que la marisma natural presenta una mayor proporción de carbono aromático y los resultados de pirólisis analítica señalan una mayor contribución de compuestos microbianos. Estas diferencias ponen de manifiesto que, a pesar del largo periodo de regeneración (más de cuarenta años), la marisma regenerada no ha recuperado totalmente sus características naturales.

Palabras clave: marisma, materia orgánica edáfica, pirólisis analítica, resonancia magnética nuclear de ^{13}C en estado sólido, *Halimione portulacoides*.

Abstract

Three adjacent areas in the Urdaibai Estuary (NW Spain) comprising a reclaimed salt marsh, a natural salt marsh and a regenerated salt marsh were sampled in order to study processes and changes in organic matter (OM) composition related to reclamation and regeneration of soils and sediments in estuarine environments. For this, a general analytical description of the soil and sediment profiles was carried out and the OM was characterized by solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy and analytical pyrolysis (Py-GC/MS).

The reclaimed salt marsh displayed very different characteristics from the natural salt marsh throughout the whole profile, indicating an important degree of change in soil quality and, particularly, in OM composition caused by the reclamation process. At this site, characterized by oxic conditions and a lack of tidal influence, transformation of the former salt marsh to grassland resulted in inputs of OM richer in carbohydrates and with a lower contribution of aromatic compounds than the OM in the salt marsh soils. On the other hand, the edaphic characteristics of the regenerated salt marsh were similar to

those of the natural salt marsh and the vegetation cover was also the same. However, the natural salt marsh accumulated twice the amount of organic carbon at the surface as the regenerated salt marsh. Furthermore, the natural marsh contained more aromatic organic materials, as detected by NMR, and the Py-GC/MS results indicated a higher proportion of microbial material in the OM. These differences indicate that the regenerated marsh has not completely recovered its natural characteristics, despite the long period (more than forty years) since the beginning of the regeneration process.
Keywords: salt marsh, soil organic matter, analytical pyrolysis, ^{13}C nuclear magnetic resonance, *Halimione portulacoides*.

1.- Introduction

Estuarine systems, important interfaces between terrestrial and marine environments, are among the most productive ecosystems in the world, and fulfil a variety of ecological functions and roles of great importance, such as retention of pollutants, sediment removal, shoreline-erosion control, flood peak reduction and as carbon sinks and reservoirs of biodiversity (Rabenhorst, 1995; Zedler, 2000; Mitsch and Gosselink, 2000). Costanza et al. (1997) estimated the current economic value of the estuarine ecosystems to be the highest among the 16 most representative world ecosystems.

Throughout history populations have settled in coastal areas, and thus large extensions of coastal wetlands have been occupied and significantly altered by human activity. In Spain, in the last 200 years the area occupied by coastal wetlands has been reduced by nearly half, mainly as result of land reclamation. In the reclamation process, an estuarine area, commonly a salt marsh, is embanked, drained and then used for farming, grazing or urban purposes, giving rise to important transformations in the affected soils (Chapter II of this dissertation, Santín et al., 2007). The reclaimed area is commonly designated by the Dutch word “polder”.

Nowadays, widespread efforts are being made to restore and even to create salt marshes in order to replace their lost ecological functions and values (Craft et al., 2002). Some reclaimed areas are being re-opened to the sea via breaching or suppression of dykes (Armel et al., 2008) or managed realignment (Spencer et al., 2008), whereas others are recovering their natural conditions by the gradual abandonment of land-use and/or the accidental breaching of sea walls (Cearreta et al., 2002; Fernández-Iglesias and Fernández-Alonso, 2008).

Numerous studies have evaluated the changes caused by land reclamation in salt marshes and estuaries (Ellis and Atherton, 2003; Portnoy and Giblin, 1997; de Jong et al., 1994) and others have investigated the changes that occur following the restoration process (Sinicrope et al., 1990: Mitsch et al., 1998; Morgan and Short, 2002). In

such studies, organic matter (OM) accumulation and content are commonly used as indicators of the restoration and/or degree of degradation (Spencer et al., 2008; Morgan and Short, 2002, Craft et al., 2003; Ellis and Atherton, 2003; Galatowitsch and vanderValk, 1996). However, changes concerning OM characteristics are often not dealt with (e.g. Craft et al., 1988; Craft et al., 2003). The composition of OM is a very fundamental topic, since it determines numerous factors such as soil quality and fertility, cycling and stabilization of carbon and nitrogen, OM humification and decomposition, adsorption of metals and pollutants, cation exchange capacity, soil aggregate stability and water infiltration (e.g. Stevenson, 1994).

The objective of the present study was to characterize OM from natural salt marsh soils and sediments and to compare it with OM from reclaimed and from regenerated salt marshes. For these purposes, three adjacent areas were sampled in the Urdaibai Estuary (Basque Country, NW Spain): a reclaimed salt marsh, a natural salt marsh and a regenerated salt marsh. A general analytical characterization of the soil and sediment profiles was carried out. Furthermore, OM was characterized by ^{13}C Variable amplitude cross polarization magic angle spinning (VACP/MAS) Nuclear Magnetic Resonance (NMR) and analytical pyrolysis coupled with gas chromatography-mass spectrometry (Py-GC/MS). The combination of both spectroscopic techniques has been used successfully in previous studies regarding OM composition in soils and sediments (Nierop et al., 2001; Quénéa et al, 2005; Simpson et al., 2005; De la Rosa et al., 2008 a, c). From the results obtained, alterations caused by the reclamation and the regeneration processes in soils and sediments are discussed, specifically those related to OM composition.

2.- Material and methods

2.1. Study area

The Urdaibai Estuary (43º 24' N 2º 41' W) is located in the Bay of Biscay, on the northwestern Coast of the Iberian Peninsula. This region has an oceanic climate with average annual precipitations of around

1200 mm and a mean annual temperature of between 13 and 14º C. The estuary is 12 km long and between 1 and 1.5 km wide and the area of the catchment basin is 183.2 km^2. The mean depth is 2.5 m and the salinity varies from 35.5% at the mouth of the estuary to 15% in the upper part. It is stratified in winter and well-mixed during summer (Indurot, 2006).

The Urdaibai Estuary maintains valuable estuarine habitats such as extensive intertidal flats and salt marshes, which support a rich diversity of flora and fauna. In 1984, it was declared a National Biosphere Reserve by UNESCO. Historically, the 1200 ha of this estuary were reduced to less than 40% by the reclamation and drainage of estuarine areas for farming, grazing or urban purposes (Gogeascoechea and Juaristi, 1997). Nowadays, salt marshes occupy a large part of the estuary (458 ha), but more than 75% of these salt marsh areas are partially or completely altered by human activities. However, changes in land-use and the decline in agriculture and other rural activities have resulted in a gradual regeneration of salt marshes, due the abandonment of land cultivation and the destruction of dams. Since the 1960s, some 110 ha of salt marshes have been regenerated and presently, more than 127 ha are subjected to this recovery process (Indurot, 2006).

2.2. Sampling

The sampling area is located in the middle of the estuary, 4 to 5 km away from the estuary mouth. Three adjacent areas in similar physiographic positions but different levels of anthropogenic influence were sampled (Figure 1. A):

A reclaimed salt marsh, “The Kanala polder” (KP): it is an active reclaimed area, mainly used for grazing and harvesting. The vegetation is predominated by grasses (e.g.: *Trifolium* sp., *Dactylis* sp., *Holcus* sp., *Cynosurus* sp.).

A natural salt marsh: this salt marsh area is dissected by a network of tidal channels and is mainly covered by the halophytic shrub *Halimione portulacoides*, an evergreen C3 Chenopodiaceae, 20 to 50 cm in height, which adapts well to drought and salinity stress (das Neves et al., 2008). This species predominates in the high salt marshes of the Urdaibai

Estuary (Indurot, 2006) and, in general, is one of the most widespread species in the high salt marshes of Europe, along with *Juncus maritimus* (Adam, 2002). Two main units were sampled in the natural marsh: a salt marsh soil covered by *H. portulacoides* (SM1) and bare sediments from a tidal channel (TC1).

A regenerated salt marsh: This polder was abandoned in the 1960s due to the abandonment of the agricultural activities in the area. Since then, the natural conditions have gradually been recovered and the area is now very similar to the nearby natural salt marsh (ii), and displays the same vegetation cover (Fig. 1. A). In the natural salt marsh, a point covered by *Halimione portulacoides* (SM2) and a tidal channel (TC2) were also sampled.

An aerial photo from 1956 (Fig. 1. B) confirms that at this time, the current regenerated salt marsh was still part of the reclaimed area and was used for agricultural purposes.

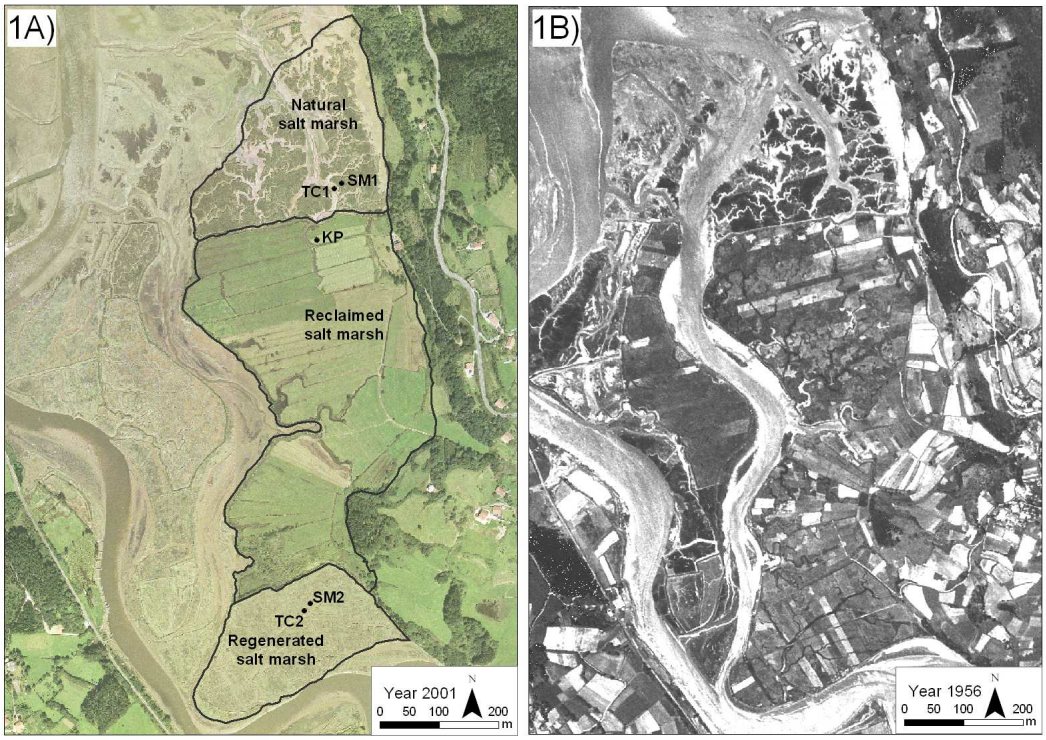

Figure 1. Location of the Urdaibai Estuary (NW Spain). 1. A) Aerial photograph from 2001 showing the sampling sites: reclaimed salt marsh (KP), natural salt marsh (SM1, TC1) and regenerated salt marsh (SM2, TC2). 1. B) Aerial photograph from 1956 showing that at that time the currently regenerated salt marsh was still under agricultural use.

Sampling was carried out in the summer 2005, during low tide. At each site, one soil profile was collected with a PVC core (11 cm i.d. and 60 cm in length). The cores were hermetically sealed, stored in an ice box at approx. 4º C, and transported in vertical position to the laboratory. The cores were then cut into 5-cm sections. Three samples were collected from each profile: one from the surface layer (0-5 cm), another from subsurface (20-25 cm) and one more from a deep layer (40-50 cm).

2.3. Determination of soil characteristics

The pH in water (soil-water ratio 1:2.5) was determined with a Crison micropH 2000 pH meter, with two buffer solutions of pH 4 and pH 7, and the electrical conductivity was measured with a Crison CM 2201 apparatus, previously calibrated with a 0.1 M KCl solution. The colour of the sample was identified by use of Munsell soil colour charts. The presence/absence of sulphide (HS^-) and metastable iron sulphides (FeS, Fe_3S_4) was tested for by addition of 6M HCl to the fresh samples; detection of a strong smell of the sulphydric acid was considered as a positive result.

The contents of oxyhydroxides-iron and pyrite-iron were determined in fresh samples (Huerta-Díaz and Morse, 1990). The former were extracted by the citrate-dithionite method and the latter with HNO_3. Prior to the extraction of pyrite-iron, the samples were mixed with 10 M HF and shaken for 16 h in order to remove iron associated with silicates. The concentration of iron in each extract was yielded by the use of flame-atomic-absorption spectrophotometry (Perkin Elmer model 1100B). The concentrations are always expressed in terms of dry weight of soil.

For further analysis, the fresh samples were dried at 55º C, passed through a 2 mm sieve and pulverized. Total carbon (TC), total nitrogen (TN) and total sulphur (TS) were measured in duplicate by dry combustion, in an Elementar Vario MAX CNS Analyzer. After the organic carbon was eliminated from the bulk soil by combustion for 4 hours at 450º C (Cambardella et al., 2001) the remaining carbon was assigned to inorganic carbon (IC). Total Organic Carbon (TOC) was then calculated as the difference between TC and IC, after prior correction for weight loss.

2.4. ^{13}C VACP/MAS NMR spectroscopy

Prior to solid-state ^{13}C NMR analysis, bulk soil and sediment samples with less than 5% of TOC were treated with hydrofluoric acid (HF) in order to concentrate the OM and to remove paramagnetic material (Gonçalves et al., 2003). After this treatment, the TOC content was enriched by a factor of 1.3-3.7 and the TN content by a factor of 1.9-4.1.

The free Particulate Organic Matter (f-POM) fraction of the surface samples was also characterized by ^{13}C VACP/MAS NMR. For isolation of the f-POM, 30 grams of dried soil/sediment were placed in a centrifuge tube and 150 ml of sodium hexametaphosphate, of density 1.8 g cm^{-3}, were added. The tube was shaken by hand, and the solution was centrifuged at 3000 rpm for 10 min. Thereafter, the supernatant was vacuum filtered (0.2 µm).

Thereafter, the supernatant was vacuum filtered (0.2 µm) to recover the sodium hexametaphosphate solution. The latter was reused for a second treatment of the soil/sediment. Finally, the f-POM, retained on the 0.2 µm filter, was washed with distilled water until the electrical conductivity was lower than <5µS. The f-POM was recovered from the filter by applying ultrasound, and was then freeze-dried.

^{13}C Variable amplitude cross polarization magic angle spinning (VACP/MAS) Nuclear Magnetic Resonance (NMR) was performed on a Bruker DSX 200 spectrometer operating at a ^{13}C resonance frequency of 50.32 MHz. The samples were confined in a zirconium oxide rotor with an external diameter of 7 mm. The variable amplitude cross polarization magic angle spinning technique was applied with a contact time of 1 ms, a spinning speed of 6.8 kHz and a pulse delay of 1 s. For quantification, the spectra were subdivided into different chemical shift regions according to Knicker and Lüdemann (1995): alkyl C (0-45 ppm), *N*-alkyl/methoxyl C (45-60 ppm), O-alkyl C (60-110 ppm), aromatic C (110-160 ppm), carboxyl/amide C (160-185 ppm) and carbonyl C (185-245 ppm). The relative intensity distribution of ^{13}C was determined by integrating the signal intensity in different chemical shift regions with an integration routine supplied with the instrument software. Corrections concerning the intensity distribution of spinning side bands were applied (Knicker et al.,

2005). On the basis of these data, the degree of aromaticity (%) was calculated (aromatic C * 100 / (alkyl C + *N*-alkyl/methoxyl C + *O*-alkyl C + aromatic C)) (Hatcher et al., 1981), and also the ratio of alkyl to O-alkyl C, as an indicator of the degree of OM decomposition (Baldock et al., 1997).

2.5. Py-GC/MS spectroscopy

From all the studied profiles, surface samples (0-5 cm) were also characterized by analytical pyrolysis (Py-GC/MS). This technique was performed with a double-shot pyrolyzer (Frontier Laboratories, model 2020) directly connected to a GC/MS system (Agilent 6890). Depending on the carbon content, between 0.5 to 1 mg of sample were placed in small platinum capsules. The detector consisted of an Agilent 5973 mass selective detector and mass spectra were acquired with a 70 eV ionizing energy. The GC was equipped with a fused silica capillary column HP 5 MS (30 m X 250 µm X 0.25 µm film thickness). The samples were heated at pyrolysis temperature, 500º C for 1 min. The carrier gas used was helium, with a controlled flow of 1 mL min^{-1}. The oven temperature was programmed from 40 to 100°C at 30°C min^{-1} and to 300°C at 6°C min^{-1}. The identification of individual compounds was achieved by single ion monitoring for different homologous series, low-resolution mass spectrometry and comparison with published and stored data (NIST and Wiley libraries).

3.- Results and discussion

3.1. General characterization of soil and sediment profiles

The general parameters analysed in soils and sediments are presented in Table 1. The pH values ranged from slightly acid to slightly neutral and were quite constant throughout the same profile. The highest values were observed in the tidal channels (TC1, TC2) (7.3-8.2), the natural and the regenerated salt marshes (SM1, SM2) showed similar pH ranges, between 6.4 and 6.8, and the lowest value was observed in the upper layer of the reclaimed salt marsh profile (KP) (5.5). The latter may be explained by the existence of dams blocking the tidal influence, which leads to better aeration of the soil and to the settlement of non-halophytic

vegetation. The low electrical conductivity (0.1 dS m^{-1}) confirms the lack of tidal influence in KP, and the brownish colour of the soil matrix indicates oxic conditions throughout this profile (Table 1). In the deepest part of the profile, the existence of pyrite-iron (84.7 µmol g^{-1}) and the relatively high TS content (Table 1) may be considered as remnants of the previous salt marsh environment (Chapter II of this dissertation, Santín et al., 2007).

The salt marsh soils, both natural (SM1) and recovered salt marshes (SM2), were light in colour (e.g. 5Y 4/2, olive grey), which is characteristic of suboxic conditions when part of the iron is present in the reduced form (ferrous iron) (Otero and Macías, 2002, 2003). The tidal channels (TC1 and TC2) ranged in colour from black to dark grey, typical of regularly flooded substrates with reduced conditions (Megonigal et al., 1993; Otero and Macías, 2003, Fiedler and Sommer, 2004). The reduced conditions were also confirmed by the presence of sulphide compounds in both profiles (Table 1). However, the content of pyrite-iron was much higher in the natural tidal channel (TC1) (69.4-558.6 µmol.g^{-1}) than in the recovered tidal channel (TC2) (0.2-1.4 µmol.g^{-1}), which indicates different geochemical conditions in the channels, with TC1 corresponding to an anoxic-sulphidic environment and TC2 to an anoxic environment.

Total organic carbon (TOC) decreased with depth in all profiles and ranged between 9.2 mg and 67.0 g^{-1}. The highest TOC contents were found in the surface layer (0-5 cm depth) of the salt marshes and the reclaimed marsh, and the lowest contents in the tidal channel sediments (Table 1). The natural salt marsh (SM1) accumulated twice the amount of TOC (67.0 mg g^{-1}) at the surface than the regenerated salt marsh (SM2, 31.4 mg g^{-1}). However, both profiles registered similar TOC contents at other depths. At a depth of between 20 to 25 cm, the TOC content in the reclaimed marsh (KP) was half that in the salt marsh profiles, possibly because of the difference in the vegetation cover. The polder vegetation, which mainly comprises grass, contains more superficial roots than the salt marsh vegetation, the roots of which can reach 25 cm depth (e.g. Caçador et al., 2004).

Table 1. General data from the sampled soils and sediments. Matrix colour according to the Munsell soil colour charts.

		Vegetation	Depth (cm)	Matrix Colour	pH	Conductivity (dS m^{-1})	Sulphide presence	Oxyhydroxides-Fe (µmol g^{-1})	Pyrite-Fe (µmol.g^{-1})	TS (mg g^{-1})	IC (mg g^{-1})	TOC (mg g^{-1})	TOC/NT
Reclaimed Salt Marsh	Kanala Polder (KP)	grasses	0-5	10YR 3/2	5.5	0.1	-	425.0	1.2	1.1	0.3	66.0	11.2
			20-25	10YR 5/6, 2,5Y 4/1	7.1	0.1	-	508.3	0.1	0.4	0.3	12.4	8.4
			40-50	10YR 5/6, 2,5Y 4/1	6.9	0.1	-	223.9	84.7	7.0	0.2	14.2	9.1
Natural Salt Marsh	Salt marsh (SM1)	*Halimione portulacoides*	0-5	5Y 4/2	6.4	n.d	-	501.3	1.6	2.8	0.3	67.0	13.6
			20-25	2,5Y 5/4	6.4	n.d	-	509.3	0.3	1.3	0.2	23.0	8.9
			45-55	5Y 5/2	6.5	n.d	-	604.8	0.1	1.5	0.2	12.2	8.9
	Tidal Channel (TC1)	bare sediments	0-5	Gley 2,5/N	8.2	4.2	+	401.6	69.4	7.0	0.5	14.0	10.8
			20-25	Gley 4/N	8.0	3.6	+	94.0	558.6	30.8	0.2	19.1	11.5
			40-50	5Y 4/1	7.9	2.4	-	128.0	108.5	9.5	0.2	10.3	8.4
Recovered Salt Marsh	Salt marsh (SM2)	*Halimione portulacoides*	0-5	5Y 5/3	6.7	7.9	-	422.8	1.4	1.9	0.4	31.4	10.3
			20-25	5Y 4/2	6.6	5.1	-	388.7	0.2	1.8	0.3	27.3	10.7
			40-50	5Y 4/2	6.8	0.2	+	348.1	0.4	1.3	0.2	15.6	8.7
	Tidal Channel (TC2)	bare sediments	0-5	Gley 2,5/N	7.6	5.0	+	345.9	2.8	2.4	0.3	11.8	10.9
			20-25	Gley 4/10Y	8.0	0.2	+	260.8	0.3	1.4	0.3	10.3	8.5
			50-60	Gley 3/N	7.3	2.2	+	349.6	0.2	0.8	0.2	9.2	9.9

n.d: not determined

Inorganic carbon contents were very low throughout the profiles (0.5-0.2 mg g^{-1}). The TOC/TN ratio ranged between 8.4 and 13.6, with the highest values generally found at the surface, but there were no clear trends with depth or among similar sites.

Taking into account most of the parameters analyzed, three groups of environments can be defined: i) the reclaimed salt marsh soil (KP) with oxic conditions, low pH and conductivity and high TOC accumulation only in the first centimetres of the soil; ii) the salt marsh soils (SM1, SM2) with suboxic conditions and high TOC contents; iii) the tidal channel sediments (TC1, TC2), which present anoxic or anoxic-sulphidic conditions throughout the whole profile, high pH, and low TOC content.

3.2. Organic matter characterization by ^{13}C VACP/MAS NMR

3.2.1. Bulk soils and sediments

The ^{13}C VACP/MAS NMR spectra of the soil and sediment profiles are shown in Fig. 2 and the ^{13}C intensity distributions (%) are summarized in Table 2.

The KP profile displayed very fresh organic material at the surface, and the distribution of ^{13}C in the spectrum was found to be very similar to that observed in other grassland soils (Helfrich et al., 2006) with low aromatic (14.6%) and aliphatic C contents (21.4%) and a predominance of O-alkyl compounds (110-60 ppm), mostly derived from carbohydrates. The small peak at 102 ppm indicated that some of the carbohydrate material is in the polysaccharidic form (Preston, 1996). The O-alkyl region constituted 43.1% of the TOC of the surface sample (0-5 cm) and this decreased to 30.4% of TOC in the deepest layer (40-50 cm). The degree of aromaticity was low throughout the whole profile, and increased with depth, from 16.4% at 0-5 cm to 25.0% at 40-50 cm. An increasing trend was also observed for the relative contribution of aliphatic C (0-45 pp) which accounted for 21.4% of TOC at the surface and reached 26.9% of TOC at depth. In the surface sample, a peak at 21 ppm supports the existence of short-chain alkyl structures attributable to methyl groups - probably in lipids, hemicelluloses (Kögel-Knabner, 2002) or peptides structures

(Knicker, 2000). The resolution of the signal at 21 ppm decreased with increasing depth, and the peak shifted towards 30 ppm. Thus, at depth the alkyl C region (0-45 pp) was dominated by a signal peaking at 28-30 ppm, typical of the internal methylene C in long alkyl chains, which may indicate preservation of long alkyl chains from plant waxes such as cutin or suberin (Preston, 1996). According to this, the alkyl/carboxyl ratio increased with depth, from 2.4 at the surface to 3.1 at depth, which suggests the presence of peptidic material at the surface and a predominance of long alkyl chains at depth (Knicker et al., 1996). Furthermore, the increase in the alkyl/O-alkyl ratio with depth (Table 2) indicates degradation of the OM through the profile. This was expected, since polysaccharides are the organic compounds most susceptible to degradation and the simultaneous increase in the proportion of alkyl C may be attributable both to selective preservation of plant biopolymers (Golchin et al., 1996) and to in situ synthesis (Baldock et al., 1990).

Table 2. ^{13}C intensity distribution (%) in the VACP/MAS ^{13}C NMR spectra of the soil and sediment profiles and of the free Particulate Organic Matter fraction of the surface layers (0-5 cm). Ar = aromaticity (%) and Alkyl C/ O-Alkyl C ratio.

		Chemical shift range (ppm)							
		245-185	185-160	160-110	110-60	60-45	45-0		
Sampling Point	Depth (cm)	Carbonyl	Carboxyl/ amide	Aromatic	O-Alkyl	N-Alkyl/ methoxyl	Alkyl	Ar. (%)	Alkyl/ O-Alkyl
	0-5	2.6	8.8	14.6	43.1	9.6	21.4	16.4	0.4
	20-25	4.4	8.4	21.2	35.8	8.2	21.7	24.4	0.5
KP	40-50	4.2	8.7	21.8	30.4	8.1	26.9	25.0	0.7
	0-5	3.1	9.8	22.2	38.4	8.8	17.6	25.6	0.4
	20-25	1.2	8.0	18.2	39.1	10.7	22.8	20.1	0.5
SM1	45-55	6.2	9.8	25.7	25.5	7.4	25.5	30.6	0.8
	0-5	3.6	9.3	26.4	26.0	9.7	25.1	30.3	0.7
	20-25	2.2	7.1	27.5	26.1	11.1	26.1	30.3	0.7
TC1	40-50	3.6	7.2	30.9	26.2	9.1	23.1	34.6	0.7
	0-5	2.9	7.9	18.6	35.4	10.0	25.2	20.8	0.6
	20-25	2.5	8.8	20.0	33.0	10.0	25.7	22.5	0.6
SM2	40-50	2.8	9.9	21.3	31.5	10.1	24.3	24.4	0.6
	0-5	2.4	7.4	21.9	29.0	9.8	29.6	24.2	0.8
	20-25	1.2	8.1	27.5	28.5	8.2	26.4	30.3	0.7
TC2	50-60	3.0	8.2	30.6	23.4	7.9	26.9	34.4	0.9
Free Particulate Organic Matter									
KP	*0-5*	*3.2*	*5.8*	*16.2*	*52.0*	*7.8*	*15.1*	*17.8*	*0.3*
SM1	*0-5*	*3.2*	*6.6*	*18.8*	*45.7*	*9.1*	*16.6*	*20.9*	*0.3*
TC1	*0-5*	*4.6*	*8.4*	*47.5*	*17.7*	*5.9*	*15.9*	*54.6*	*0.7*
SM2	*0-5*	*4.7*	*7.1*	*21.9*	*43.7*	*8.9*	*13.7*	*24.9*	*0.3*
TC2	*0-5*	*4.5*	*7.5*	*28.0*	*35.1*	*8.0*	*16.8*	*31.8*	*0.4*

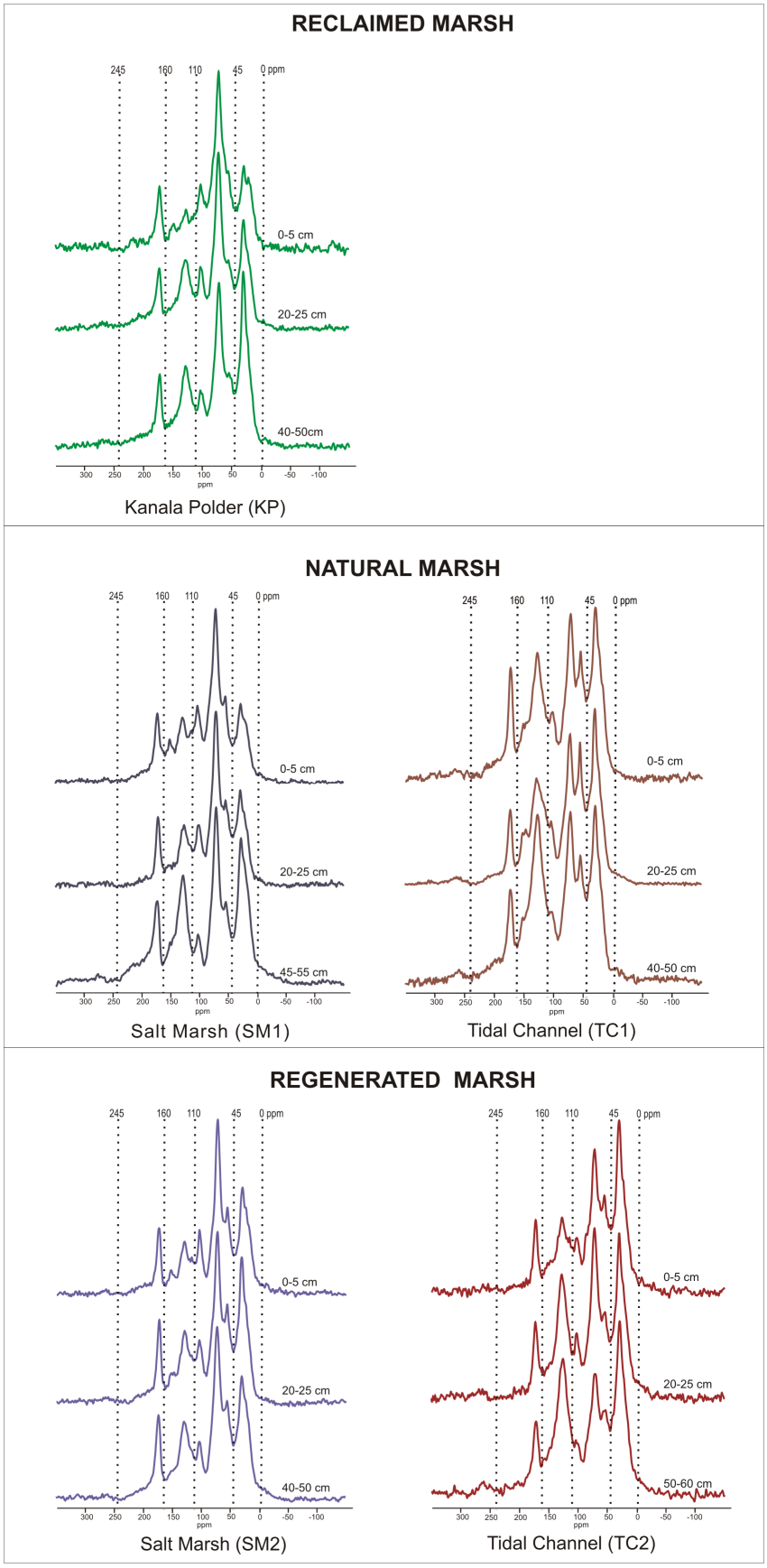

Figure 2. Solid-state ^{13}C VACP/MAS NMR spectra of the soil and sediment profiles.

Corresponding sites from the natural and regenerated salt marshes sites (SM1 and SM2) displayed similar ^{13}C distribution in the NMR spectra, indicating comparable OM composition, although some differences were also detected (Fig. 2). The peak at 72 ppm dominated in all spectra. This peak was mainly attributable to carbohydrate-derived structures. A smaller peak at 102 ppm was also detected, confirming the presence of polysaccharides through the profiles. The contribution of aromatic compounds was notable and, in conjunction with the signal at 55 ppm, suggests the existence of lignin (Kögel-Knabner, 2002). The highest degree of aromaticity was observed at depth in both profiles, and was higher in SM1 (30.6%) than in SM2 (24.4 %). Also at surface, the degree of aromaticity was higher in the natural salt marsh (25.6%) than in the regenerated salt marsh (20.8%). This may be related to the higher accumulation of OM in the former (see section 3.1), since aromatic compounds are more stable and more resistant to degradation. Another remarkable difference was the high aliphatic C content in the regenerated salt marsh (SM2) at surface (25.2%).

Finally, some similarities were detected on comparing the two tidal channel spectra (TC1 and TC2) (Fig. 2). Furthermore, it is interesting to note that both profiles have a different OM composition from that typically attributed to estuarine or marine sediments where the alkyl compounds clearly predominate (e.g.: Gélinas et al., 2001; Hedges and Oades, 1997). Although both profiles have a higher alkyl C content (from 23.1% to 29.6%) and lower contribution of polysaccharides than the other profiles (from 23.4 to 29.0%), they still show a large contribution of aromatic compounds, which increases with depth. Indeed, they display the highest degree of aromaticity of all the studied profiles (Table 2). The high intensity in the region of aromatic C (110-160 ppm) of their solid-state ^{13}C NMR spectra, and the clear signal at 55 ppm, assignable to methoxyl C, suggest that a large fraction of the organic matter is derived from lignin containing vascular plant compounds (Kögel-Knabner, 2002). Nevertheless, aromatic C may also originate from black carbon residues (Preston and Schmidt, 2006).

3.2.2. Free particulate organic matter

The free POM fraction is mainly constituted of relatively fresh OM inputs (e.g. plant residues in soil) that yet are not interacting with the mineral phase. It has been suggested that this reflects the most recent OM contributions to soil and sediment (Golchin et al., 1994). Thus, in order to characterize the current OM inputs, we concentrated on analysis of the surface layers (0-5 cm). The ^{13}C carbon distribution of the f-POM in the different samples is shown in Table 2 and their ^{13}C VACP/MAS NMR spectra in Figure 3.

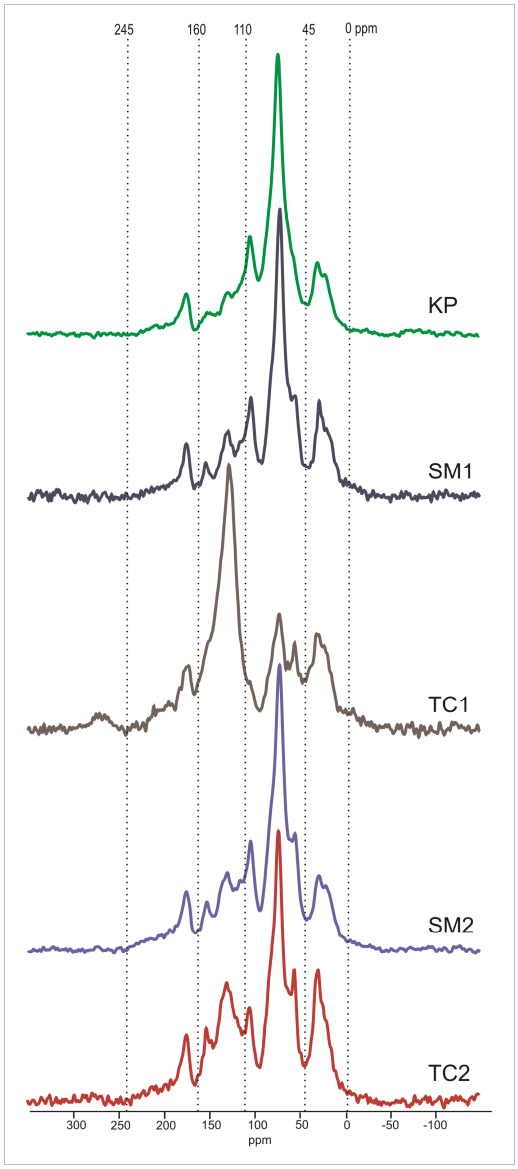

Figure 3. Solid-state ^{13}C VACP/MAS NMR spectra of the f-POM of surface soils and sediment (0-5 cm).

The free POM in the reclaimed marsh (KP) is dominated by the signal in the O-alkyl C region (52% of the total intensity) which can be assigned to polysaccharidic compounds. The composition of the f-POM in the two salt marshes covered by *Halimione portulacoides* (SM1 and SM2) is similar to that of KP and mainly comprises O-alkyl C (45.7% in SM1 and 43.7% in SM2). However, the contributions of aromatic C to the f-POM are more important than in KP (Table 2). This is probably related to the different type of vegetation cover since a woody plant such as *H. portulacoides* contributes more lignin to the soil OM than the grasses of the polder (Higuchi, 1997). According to the results of NMR spectroscopy, the f-POM composition in the tidal channels (TC1, TC2), is completely different from that observed for the other samples (Table 2), reflecting a high degree of aromaticity concomitant with the low contribution of O-alkyl C (Fig. 3). In contrast to many examples of sedimentary organic matter (Hedges and Oades, 1997), the alkyl-C region comprises only 15.9% and 16.8% of the organic C of the f-POM of TC1 and TC2, respectively. Especially in TC1, the great intensity of the aromatic C signal (Fig. 3) suggests the predominance of black carbon or charred materials (Preston and Schmidt, 2006).

3.3. Organic matter characterization by Py-GC/MS

The total ion current (TIC) traces and released compounds obtained by GC/MS analysis of the pyrolysates of the surface samples of all studied profiles are reported in Figure 4, and a list of the compounds identified is shown in Table 3. Because many compounds released with the pyrolysates have a unique origin, the evolved compounds in the pyrolysis of samples can be used as a “fingerprint” of a specific sample or changes that have occurred (Almendros et al., 1997).

The TICs reveal the presence of alkane/alkene pairs (A-11 to A-32 in Table 3), of molecules derived from carbohydrates (C1 to C16), of linear fatty acids (FA12 to FA26), of compounds of peptidic origin (P1 to P18), and of lignin pyrolysis products (L1 to L7), together with a series of 40 compounds without a unique origin. The latter mainly consists of benzene derived compounds (including phenols, alkylbenzenes and polycyclic

aromatic hydrocarbons), akylnitriles and sulphurated compounds (Table 3). Among the identified signals (Fig. 4), those with the greatest intensities correspond to carbohydrate pyrolysis products (C2, C4, C7). Phenol (9) and cresol (10 and 11) compounds are also abundant in all the samples but their origin is very variable; they can derive from cellulose (Pastorova et al., 1994), tyrosine (Stankiewicz et al., 1996) or severely altered lignin derivatives (Saiz-Jimenez and de Leeuw, 1986a).

The pyrogram of the reclaimed marsh sample (KP) shows remarkable differences from the other samples studied (Figure 4). There was a notable presence of fatty acids (FA) in this sample. FA are ubiquitous in the biosphere, and are found in algae, bacteria and other microorganisms and terrestrial plants. FA from plants have a predominance of even over odd-numbered chain lengths (Meyers, 1997). C12 FA is present in this sample, which is presumably of microbial origin, and also C16-C18 FA, which are common to both plants and microorganisms (Buurmann et al., 2007). However, the notable presence of longer chain C22-C26 homologues (Table 3) is related to the wax coating and plant-derived sources (Almendros et al., 1996). In addition, the KP pyrogram revealed a wider variety of polysaccharides compounds (see peaks C16 and C17) and a relative enrichment of some lignin derived compounds (e.g. L3 in Fig. 4) than the other samples. Despite the fact that most lignin derived compounds are not detectable by flash pyrolysis-GC/MS because they decarboxylate at high temperatures (Martín et al., 1994), their presence suggests a strong contribution of plant inputs. More specifically, the presence of methylphenols (Table 3) in addition to guaiacyl derivatives (L2 and L3) indicates a predominance of grass lignin (Saiz-Jimenez and de Leeuw, 1986b). Moreover, the presence of styrene in this pyrogram (peak 39) is also remarkable. This compound is thought either to derive from aromatic residues of anthropogenic input, degraded lignin, tannins or as a residue derived from thermally altered OM (van Smeerdijk and Boon, 1987). Taking into account that styrene has not been detected in any of the adjacent natural/regenerated sites, its presence may be associated with an anthropogenic source in the reclaimed marsh. Therefore, the observed differences in the KP pyrogram may indicate that

alteration of the OM composition was caused by the reclamation process and the consequent changes in land-use and vegetation cover.

As regards the natural and regenerated salt marshes (SM1 and SM2), although both samples exhibit rather similar pyrograms, several differences were detected. Both samples are rich in carbohydrate-derived compounds (Fig. 4); moreover, the presence of some linear long chain FA and a relative enrichment in long n-alkanes/enes also indicate land plant-derived OM sources. Interestingly, as regards lignin derived compounds, a wider variety was observed in SM1 (L1-L7) than in SM2 (L1, L3 and L5). Along with the fact that SM1 presented a higher TOC/N ratio (Table 1) and a higher degree of aromaticity (Table 2), this suggests a greater contribution of lignin derived compounds in SM1 than in SM2.

Furthermore, straight-chain alkenes/alkanes are commonly found in pyrolysates of plant materials and soils (Kögel-Knabner et al., 1992), whereas branched pairs have rarely been detected (Nierop et al., 2001). The fact that branched alkanes were detected in the three soil samples (KP, SM1, SM2 in Fig. 4) may indicate microbial activity in these soils, with the greatest intensities and number of branched alkanes detected in SM1 (Fig. 4). In agreement with this, there was also a predominance of even over odd-numbered chains in the alkanes/alkenes series in SM1 (see A-22 to A-30, Fig. 4) which also indicates microbial contributions (Grimalt and Albaigés, 1987). In the SM2 pyrogram the predominance of even numbered chains in the alkanes/alkenes series was not as clearly expressed.

Comparison of the tidal channels (TC1 and TC2) with the adjacent marshes (SM1 and SM2, respectively) reveals a loss of the predominance of even numbered chains in the series of *n*-alkanes in the tidal channels, which indicates different origin of the OM inputs. Any branched alkanes are detected in the sediment samples. Furthermore, a relative enrichment of sulphurated compounds was observed in both tidal channels (TC1 and TC2) (see peaks 1 and 7 in Fig. 4), which is consistent with the presence of sulphide compounds registered in these sediments (Table 1). Moreover, they presented the lowest amount of pyrolysable material, which is also consistent with higher contributions of pyrogenic organic matter (De la

Rosa et al., 2008c) and with the ^{13}C VACP/MAS NMR observations (see section 3.2). This conclusion is supported by signals assignable to polycyclic aromatic hydrocarbons (PAHs) (peaks 17, 20, 26, 29 or 30 in Fig. 4), which in non-polluted soils and sediments, have been described as markers of black carbon or charred material (De la Rosa et al., 2008c).

The absence of lignin derived compounds in the pyrogram of the TC1 sample is remarkable, and suggests a low or no input of fresh litter from terrestrial vegetation, or that the latter has been highly degraded. The recovery of polysaccharides is also relatively low and the n-alkane distribution, with no significant predominance of high molecular weight homologues, may be related to diverse sources such as marsh vegetation (Simoneit et al., 1984), aquatic macrophyta, microalgae (Ficken et al., 2000) or sea grasses (Jaffé et al., 2001). Nevertheless, the accumulation of low molecular weight homologues, which suggest the occurrence of thermal break down and cracking of long chain components, and the decrease in the normal dominance of odd numbered alkanes have also been reported to be indicative of fire events (Almendros et al., 1988; González-Perez et al., 2008). The latter statement is also consistent with the high aromatic content detected by NMR spectroscopy in this sample (section 3.2), and could explain the absence of lignin derived compounds.

On the other hand, the pyrogram corresponding to the TC2 sample shows the presence of lignin derived compounds, which together with the predominance of n-alkanes with an odd number of carbons (A-25 to A-31) and a maximum in the A-27, indicates a considerable contribution of fresh vascular plant inputs to this OM. It has been reported that those n-alkanes originate from higher plants waxes (Eglinton and Hamilton, 1963, Jaffé et al., 1995), and it has also been found that a series of n-alkanes with a maximum between C23-C27 correspond to typical marsh vegetation (Simoneit et al., 1984).

Table 3. Compounds released by pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS) from the studied samples.

Assignment	Peak code
SO_2	1
benzene	2
acetic acid	3
toluene	4
n,n '-dimethylbenzene	5
heptanenitrile	6
thiophenecarboxaldehyde	7
phthalate	8
phenol	9
o -cresol	10
p -cresol	11
naphthalene	12
phenylacetone	13
benzylnitrile	14
p -xylenol	15
o -ethylphenol	16
1-methylnaphthalene	17
benzenepropanenitrile	18
lemonene	19
n,n´ -dimethylnaphthalene	20
2-heptyl-thiophene	21
n,n',n''-trimethylnaphthalene	22
9H-fluorene	23
pentadecanenitrile	24
dodecylbenzene	25
phenanthrene	26
hexadecan-2-one	27
heptadecanenitrile	28
1-methylanthracene	29
2-methylanthracene	30
hexadecylbenzene	31
nonadecanenitrile	32
fluoranthene	33
2-methylbutanal	34
propanoic acid	35
2-nonadecanone	36
1-hexadecanol	37
pentacosanenitrile	38
styrene	39
1-octadecanol	40
dodecanoic acid	FA12
pentadecanoic acid	FA15
hexadecanoic acid	FA16
octadecanoic acid	FA18
nonadecanoic acid	FA19
eicosanoic acid	FA20
docosanoic acid	FA22
tricosanoic acid	FA23
tetracosanoic acid	FA24
pentacosanoic acid	FA25
hexacosanoic acid	FA26
guaiacol	L1
p -ethylguaiacol	L2
p -vinylguaiacol	L3
syringol	L4
isoeugenol	L5
acetovanillone	L6
methoxyeugenol	L7

Assignment	Peak code
2-methylfuran	C1
furfural	C2
2,5-dimethylfuran	C3
5-methyl-2-furfural	C4
benzaldehyde	C5
2-methylciclopenten-1-one	C6
2,4-dimethylfuran	C7
2(5H)-furanone	C8
2,3-dihydrobenzofuran	C9
4-methyldibenzofuran	C10
3-methylfuran	C11
2-acetylfuran	C12
octylfuran	C13
methylcyclopentenone	C14
D-allose	C15
nonylfuran	C16
pyridine	P1
1H-pyrrole	P2
n -methyl-pyridine	P3
n -methyl-1H-pyrrole	P4
n -ethylpyridine	P5
1-H-pyrrole-carboxaldehyde	P6
2-pyridinecarbonitrile	P7
3-acetylpyrrole	P8
benzopyridine	P9
Indole	P10
n -carbonitrile-5-methyl(1H)pyrrole	P11
3-phenylpyridine	P12
2-methyl-1H-Indole	P13
2-methyl-hydroxypyrone	P14
1,3-pyrrolidinedione	P15
2-phenylacetonitrile	P16
L-leucine	P17
indanone	P18
undecane/ene pair	A-11
docecane/ene pair	A-12
tridecane/ene pair	A-13
tetradecane/ene pair	A-14
pentadecane/ene pair	A-15
hexadecane/ene pair	A-16
heptadecane/ene pair	A-17
octadecane/ene pair	A-18
nonadecane/ene pair	A-19
eicosane/ene pair	A-20
heneicosane/ene pair	A-21
docosane/ene pair	A-22
tricosane/ene pair	A-23
tetracosane/ene pair	A-24
pentacosane/ene pair	A-25
hexacosane/ene pair	A-26
heptacosane/ene pair	A-27
octacosane/ene pair	A-28
nonacosane/ene pair	A-29
triacontane/ene pair	A-30
henetriacontane/ene pair	A-31
dotriacontane/ene pair	A-32

Note: Peak labels FA, L, C, P and A- refer to molecules derived from fatty acids, lignin, carbohydrates, peptides and alkanes/alkenes respectively.

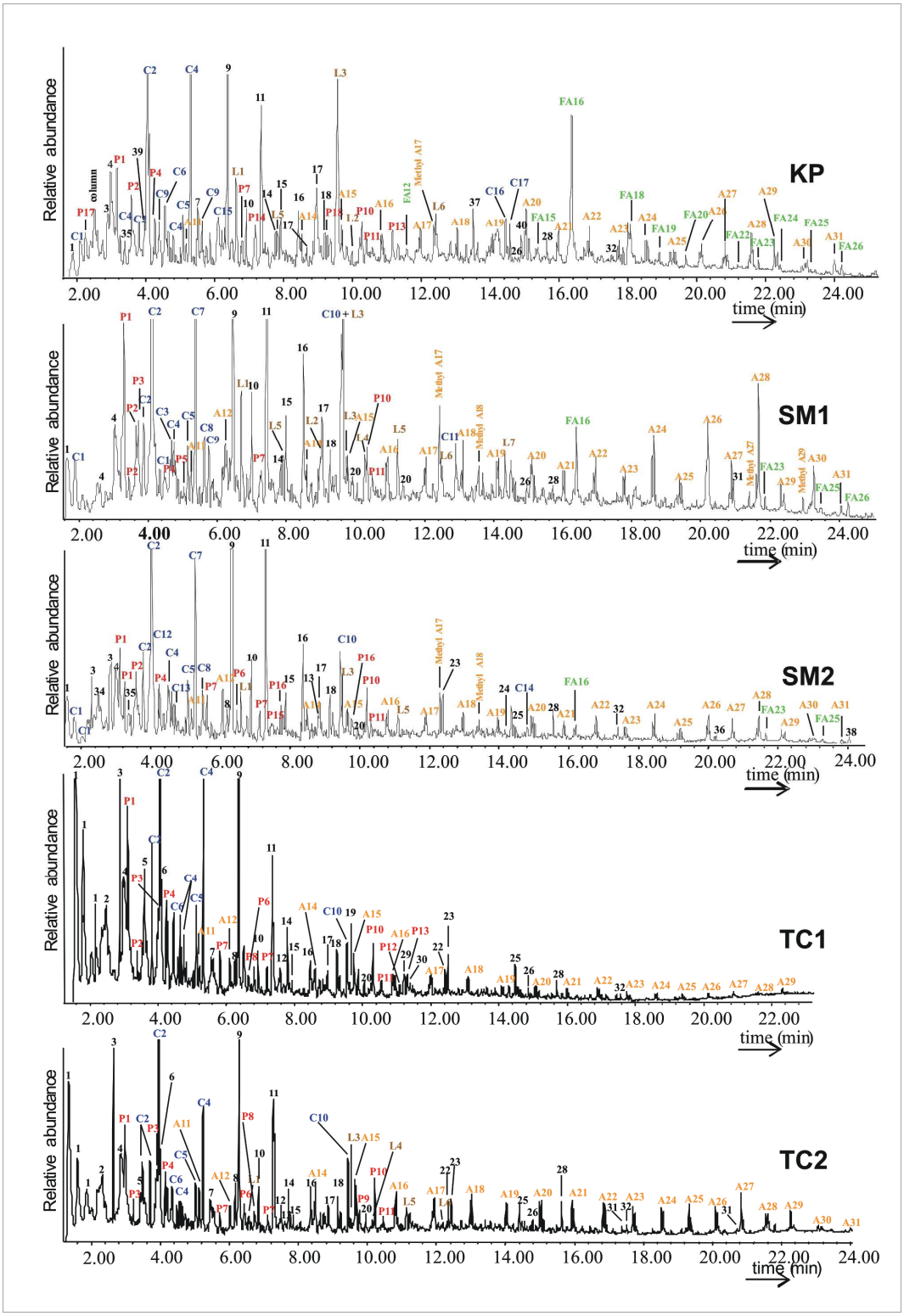

Figure 4. Total ion chromatograms (Py-GC/MS). Numbers on the peaks refer to Table 3.

3.4. Organic matter changes attributed to reclamation and regeneration processes

Determination of general soil parameters of the sampled profiles, along with characterization of their OM by means of ^{13}C VACP/MAS NMR and Py-GC/MS show that a wide variety of soil and sediment characteristics can be found within the estuarine environment at a reduced spatial scale. In these land to sea transitional environments a large number of factors control the soil and sediment characteristics: high-scale factors such as climate or geology; medium-scale factors such as vegetation cover or physiography, and micro-scale factors such as the geochemical environment (e.g.: Chapters III and IV of this dissertation; Goñi and Thomas, 2000; Álvarez-Rogel et al., 2006; Santín et al., 2008 a, b). The results of the present study demonstrate that anthropogenic activities can also contribute to the high variability of these estuarine systems, and that they can cause noteworthy alterations.

The present results indicate that the reclamation process produces a great transformation of the soil characteristics. Blockage of the tidal influence by dams leads to salt leaching and aeration of the soil, which make settlement of non-halophytic vegetation possible. Furthermore, the conversion from marsh to grassland strongly affects the quality of the soil OM. OM inputs richer in carbohydrate materials from grasses lead to a low amount of aromatic and stable OM in the soil. Although it is expected that oxic soil conditions will lead to a higher decomposition/humification of OM in salt marsh soils (Perobelli-Ferreira et al., 2008, Santín et al., 2008a), the opposite trend is observed in the present study. We attributed our results to the difference in the inputs of vegetation between the reclaimed grassland (oxic conditions) and the salt marshes (suboxic conditions), since woody plants such as *H. portulacoides,* contribute more lignin compounds to the soil OM than grasses.

Comparison of the natural and the regenerated salt marshes (SM1 and SM2) showed that the vegetation cover (*Halimione portulacoides*) is the same at both sites; characterization of the f-POM by solid-state ^{13}C NMR revealed very similar vegetal inputs. Therefore, the observed

differences, such as a greater OM accumulation and a higher contribution of aromatic compounds and microbial-derived OM in SM1, are unlikely to be caused by different OM inputs. In the Chapter III of this dissertation has been demonstrated that in salt marsh soils OM quality can differ, even with the same vegetation cover, due to differences in the geochemical environment. However, these two profiles present very similar geochemical characteristics as revealed by the general soil parameters. Alternatively, the differences between the natural and the regenerated salt marshes demonstrate that despite the long duration of the recuperation process, the natural characteristics of the OM have not been completely recovered. These results are consistent with those of Craft et al (2003), who observed that after 28 years the size of the soil TOC pool was smaller in a constructed salt marsh than in the natural salt marsh. Furthermore, Craft et al. (2002) estimated that after marsh creation, up to 90 years may be required until the size of the natural TOC pool becomes equivalent to a natural marsh.

As regards OM in tidal channels profiles (TC1 and TC2), the characterization did not show the typical features attributed to sedimentary OM (Hedges and Oades, 1997) and large terrestrial inputs to the sedimentary OM were observed. This finding is not surprising since these tidal channels are part of a network of channels inside the salt marsh areas (Figure 1). Furthermore, the sedimentary OM composition and the quality of the f-POM fraction differ in both tidal channels. For the natural tidal channel (TC1), NMR spectroscopy shows important contributions of aromatic compounds at the surface (0-5 cm), however, lignin-derived compounds were not detected by PyGC/MS. Thus, one plausible explanation is that the large amount of aromatic carbon mainly originates from charred material. The observation of small pieces of charred material during the sample manipulation supports this hypothesis. Furthermore, chemical oxidation with acid potassium dichromate was carried out as a fast approach of the determination of black carbon in the sediment samples (for further details see Knicker et al., 2007). The results prove the existence of a remaining recalcitrant aromatic C pool after the oxidation procedure in both tidal channels, especially in TC1 (Fig. 5), which confirms

the presence of charred material. These charred compounds probably derive from eroded material from burnt upland soils in the catchment area of the Urdaibai Estuary (Rumpel et al., 2006). The sediment supply from the catchment is partially deposited in the tidal channels, which form the drainage network of the estuary.

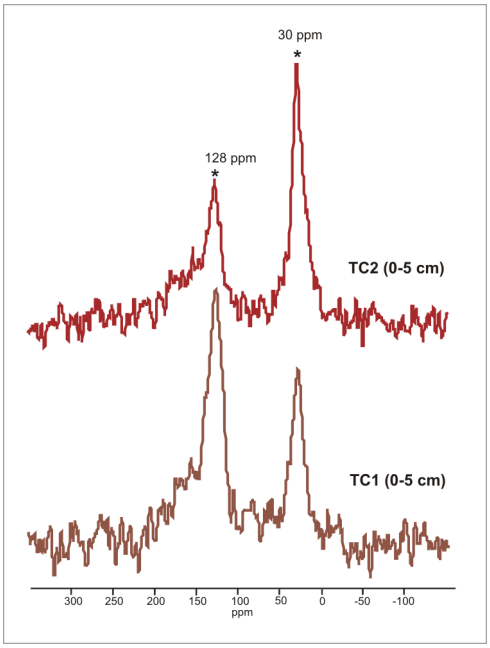

Figure 5. Solid-state 13C VACP/MAS NMR spectra of the chemical oxidation resistant elemental carbon from the surface sediment samples (6 h oxidation time). The peak at 30 ppm corresponds to paraffinic acid-resistant material, the peak at 128 ppm to small polycyclic aromatic hydrocarbons.

On the other hand, although in the natural tidal channel (TC1) the terrestrial contributions appeared to be dominated mainly by charred compounds, in the TC2 fresh inputs from vascular plants, either terrestrial or from the adjacent marsh, were detected by analytical pyrolysis. The tidal channel in the regenerated marsh (TC2) is younger, shallower and smaller, so that a different water flow dynamic would be expected, which may lead to different OM inputs. Moreover, different OM inputs may be explained by the different physiographic positions of these two tidal channels within the estuary. Further studies are required for a better understanding of the exchange processes between the estuary and the adjacent terrestrial and marine systems.

4.- Conclusions

The combination of solid state ^{13}C VACP/MAS NMR and analytical pyrolysis provided complementary windows of information about OM composition of soils and sediments from estuarine environments. The reclamation of salt marshes, which is very common practice along much of the Spanish coast, leads to substantial alteration of the edaphic characteristics and to changes in the vegetation cover with the consequent modification of the OM composition. The most significant changes are the higher contribution of polysaccharides and the lower amount and different composition of lignin-derived compounds as compared with the natural salt marsh.

On the other hand, regeneration of a salt marsh has been shown to be slow. Despite similar edaphic characteristics and the same vegetation cover, the natural salt marsh accumulated twice the amount of OM at surface as the regenerated salt marsh. Moreover, the composition of the soil OM also differed, with a larger amount of aromatic organic materials and microbial contribution to the soil OM in the natural salt marsh.

Interestingly, large amounts of recalcitrant aromatic materials were found in the tidal channel sediments, which may correspond to charred materials derived from upland environments in the catchment area of the Urdaibai Estuary.

In previous studies organic matter has been recognized as an excellent descriptor of the structure and functions of salt marshes (Craft et al., 2003). Thus, in order to identify the effects caused by the opposing processes of reclamation and regeneration on organic matter characteristics, we must improve our understanding of the anthropogenic influence in these land to sea transitional systems.

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Capítulo VI. Caracterización de sustancias húmicas de suelos y sedimentos estuarinos mediante fluorescencia en tres dimensiones y análisis factorial paralelo

Characterizing humic substances from estuarine soils and sediments by excitation-emission matrix spectroscopy and parallel factor analysis

Resumen

En este capítulo se utiliza la combinación de espectroscopía de fluorescencia en tres dimensiones (EEM) y análisis factorial paralelo (PARAFAC) para caracterizar ácidos húmicos (AH) y fúlvicos (AF) de suelos y sedimentos de dos ambientes estuarinos en España. Cinco componentes fueros identificados y caracterizados como "tipo fúlvico", "tipo húmico", "de origen microbiano", "tipo tirosina" y "tipo triptófano" respectivamente. Estos componentes son similares a los encontrados previamente en la fracción soluble en agua de suelos y también a aquellos identificados en la materia orgánica disuelta procedente de un amplio número de ambientes acuáticos. La contribución de cada uno de estos cinco componentes difiere significativamente entre los grupos de AH y AF. El componente "tipo fúlvico" es el dominante en los AF, mientras que el "tipo húmico" lo es para los AH. El componente microbiano es más abundante en los AF que en los AH, y los componentes proteicos son, en general, más abundantes en los AH. Además, un análisis de componentes principales de la abundancia relativa de cada uno de los componentes identificados reveló diferencias significativas entre los dos estuarios, entre suelos y sedimentos y entre las diferentes profundidades estudiadas.

Los resultados muestran la combinación de EEM-PARAFAC como una potente herramienta para la caracterización de sustancias húmicas de suelos y sedimentos.

Palabras clave: ácidos húmicos, ácidos fúlvicos, estuario, suelo, sedimento, EEM-PARAFAC

Abstract

The combination of Excitation-Emission Matrix fluorescence (EEM) and Parallel Factor Analysis (PARAFAC) was shown to be a powerful tool for characterizing humic acids (HA) and fulvic acids (FA) from soils and sediments of two estuarine environments in Spain. Five components were identified and characterized as fulvic acid-like, humic acid-like, microbial humic-like, tyrosine-like, and tryptophan-like components, respectively. These components were similar to those previously found in dissolved organic matter in soils or in the water soluble fraction of soils, and also to those identified in dissolved organic matter in a wide range of aquatic environments. The contributions of each of the 5 components differed significantly between HA and FA groups. The fulvic acid-like component predominated in the FA group, whereas the humic acid-like component predominated in the HA group. Furthermore, the microbial humic acid-like component was much more abundant in FA than in HA, although both protein-like components were generally more abundant in HA than in FA. Principal components analysis (PCA) of the relative abundance of each component was carried out and revealed significant differences between the FA and HA fractions. Moreover, the PCA plots showed significant differences between the two estuaries, between sediments and soils, and

among depths, indicating that EEM-PARAFAC is useful technique for characterizing humic substances.

Keywords: humic acids, fulvic acids, estuary, soil, sediment, EEM-PARAFAC.

1.- Introduction

Humic substances (HS) are the most widespread natural organic materials in the environment. They represent up to 70-80 % of organic carbon in mineral soils and up to 60% of dissolved organic carbon in aquatic ecosystems (Stevenson, 1994; Schlesinger, 1997). HS play a variety of vital roles in carbon and nitrogen cycles (Zech et al., 1997), carbon sequestration (Hayes and Clapp, 2001), mobilization and availability of nutrients (Stevenson, 1994), metals (Wood, 1996; Yamashita and Jaffé, 2008) and pollutants (Senesi, 1992), and even interactions with organisms (Steinberg et al., 2008). It is therefore clearly important to understand the nature, composition and stability of HS. However, it is not an easy task to obtain such knowledge, as HS are complex supramolecular associations of heterogeneous molecules predominantly stabilized by weak dispersive forces and derived from the degradation and decomposition of a mixture of different biological materials (Piccolo, 2001).

Humic substances have been successfully characterized by use of different modes of single-scan fluorescence spectroscopy (i.e., emission, excitation and synchronous-scan modes) (Senesi et al., 1991; Fuentes et al., 2006). Excitation-Emission Matrix (EEM) fluorescence spectroscopy has become popular for characterizing organic materials from different environments. This technique provides an overall and complete view of all the features existing within a selected spectral range and thus provides an accurate picture of the excitation-emission wavelength pairs where the studied organic material has its maxima of fluorescence. EEM fluorescence spectroscopy has been widely used for the study of dissolved organic matter (DOM) in aquatic systems (Coble, 1996; Del Castillo et al., 1999; Kowalczuk et al., 2003; Chen et al., 2003; Yamashita and Tanoue, 2003; Maie et al., 2008). Although few EEM fluorescence spectroscopy studies have been carried out with humic substances in soil and sediments to date, EEM fluorescence spectroscopy has been used to characterize HS (Sierra et al., 2005a; Alberts and Takács, 2004a, b, Mobed et al., 1996) and their interactions with trace metals (Fukushima et

al., 1997; Provenzano et al., 2004; Plaza et al., 2005) and pollutants (Sun et al., 2007). In these studies, however, notable differences were only found between the excitation-emission matrices (EEMs) of humic and fulvic fractions, but not among samples of the same fractions.

In recent years, the interpretation of EEMs of natural organic matter by means of multivariate analysis, such as parallel factor analysis (PARAFAC) has undergone substantial improvement (Stedmon et al., 2003). This statistical modeling approach enables characterization and identification of individual fluorescent groups in the EEMs. Thus, it is particularly valuable in environmental applications where standards or reference components are not always available.

The combination of EEM and PARAFAC has been widely applied in the study of aquatic ecosystems, taking advantage of the natural fluorescence of colored DOM. For instance, it has been used to characterize DOM in waters (Stedmon et al., 2003; Cory and Mcknight, 2005; Holbrook et al., 2006), to characterize water soluble DOM in soil (Ohno and Bro, 2006; Ohno et al., 2007; Fellman et al., 2008), to characterize the specific binding capacities of fluorescent components with trace metals (Ohno et al., 2008; Yamashita and Jaffé, 2008), to classify water sources and their origin (Hall and Kenny, 2005; Hua et al., 2007), and in studies of the DOM dynamics in oceanic as well as coastal environments (Stedmon and Markager, 2005; Stedmon et al., 2007; Wedborg et al., 2007; Murphy et al., 2008; Yamashita et al., 2008).

To our knowledge, however, only one study has been carried out to characterize terrestrial HS by EEM fluorescence spectroscopy combined with PARAFAC (He et al., 2006). He et al. (2006) obtained 3 components using six International Humic Substances Society (IHSS) standards and found that one component, which provided a peak at a longer emission wavelength, was more abundant in the humic acid fraction than in the fulvic acid fractions. The difference was consistent with the findings of previous EEM studies (Sierra et al., 2005a; Alberts and Takács, 2004a, Mobed et al., 1996). Thus, EEM-PARAFAC is a potentially useful technique in the assessment of complex HS in natural environments, that is, to evaluate the similarity/dissimilarity of compositions of fluorescent

groups between fractions as well as among samples. However, although EEM-PARAFAC has not yet been applied to the characterization of HS in natural environments, it may provide additional insight into the optical properties of HS as well as HS behavior.

In the present study, EEM-PARAFAC was used to characterize humic acids (HA) and fulvic acids (FA) from soils and sediments of estuarine environments. The main objectives were 1) to evaluate the major representative fluorescent groups in humic and fulvic acid fractions and to compare the fluorescent components in HS with previously reported PARAFAC components in natural environments; 2) to test the usefulness of the technique for evaluating the similarity/dissimilarity of HS from different soils and sediments.

2.- Materials and methods

2.1. Study sites and sampling

Two estuaries on the northwestern coast of the Iberian Peninsula were selected for sampling: the Urdaibai Estuary (43º 24' N 2º 41' W) and the Foz Estuary (43º 33' N 7º 15' W). The climate in the region is oceanic with mean annual temperatures of between 13 and 14 ºC and total annual precipitation between 1000 and 1200 mm. The sample set covered a high diversity of samples from estuarine environments: different depths from different salt marsh soils, a grassland soil profile from a reclaimed salt marsh and sediment profiles from tidal channels. In the present study, the substrate colonized by vascular plants is denominated soil whereas the substrate non-colonized by rooted plants is considered as sediment (for further details see Ferreira et al., 2007).

i) Urdaibai Estuary: this estuary, which covers an area of 1200 ha, has been seriously affected by the reclamation and drainage of estuarine areas for farming, grazing and urban purposes (Gogeascoechea and Juaristi, 1997). Salt marshes still occupy an important part of the estuary, although more than 75% of them are altered by human activities. The sampling area is located in the middle of the estuary, 4 to 5 km away from the estuary mouth. The selected transect (Fig. 1.A) was: a natural salt

marsh area (one sampling point in a tidal channel sediment and another in an adjacent high marsh soil covered by *Halimione portulacoides*); a regenerated salt marsh area (one sampling point in a tidal channel sediment and another in an adjacent high marsh soil covered by *H. portulacoides*); and a reclaimed salt marsh area (one sampling point in a polder grassland soil).

ii) Foz Estuary: this small estuary (460 ha) has scarcely been affected by human activity, with only 6 % of its surface reclaimed. The sampling area is located just 1.7 km from the estuary mouth, and the selected transect (Fig. 1.B) was as follows: a sampling point in a tidal channel sediment; another in an adjacent salt marsh soil covered by *H. portulacoides*; another in a high marsh soil covered by *Juncus maritimus*; and another in an abandoned polder soil recolonized by natural vegetation (*J. maritimus*).

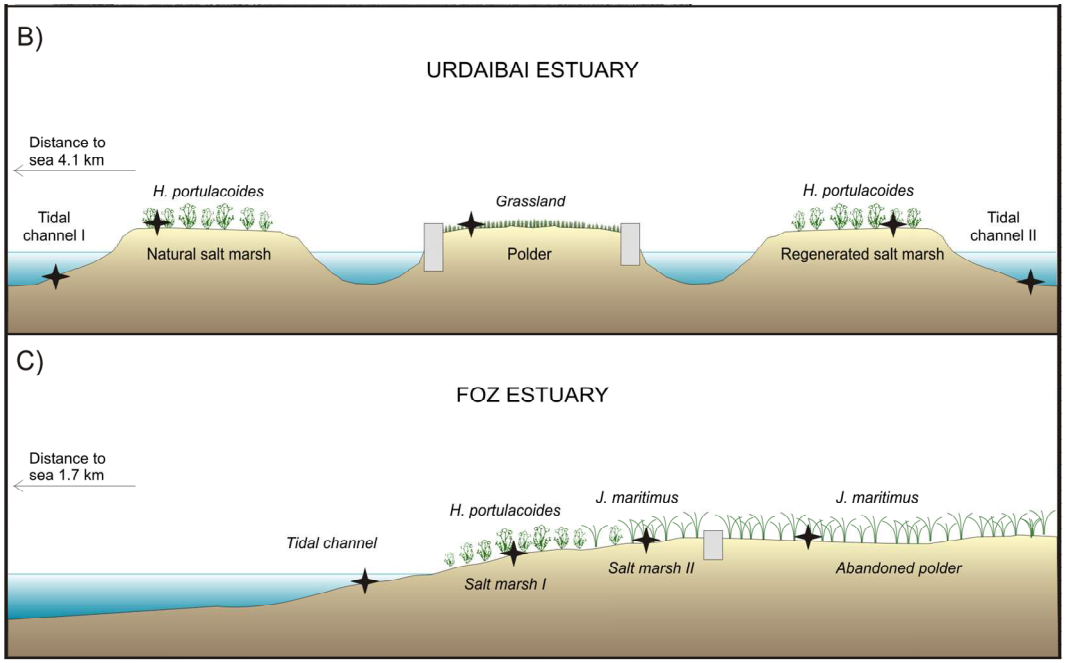

Figure 1. A) Sample locations in the Urdaibai Estuary; B) Sample locations in the Foz Estuary. Stars represent sampling points.

Sampling was carried out in the summer of 2005. Each profile was collected with a PVC core (11 cm i.d. and 60 cm in length). The cores were hermetically sealed, stored in an ice chest at 4º C, and transported in vertical position to the laboratory. The cores were then cut into 5-cm

sections. From each profile three sections were selected for the analysis: the surface layer (0-5 cm), a subsurface layer (20-25 cm) and a deeper layer (40-60 cm).

2.2. Elemental analysis

Fresh samples were dried at 55º C, then sieved through a 2 mm mesh for further analysis. Total carbon (TC), total nitrogen (TN) and total sulfur (TS) were measured in duplicate by dry combustion (975º C) in an Elementar Vario MAX CNS Analyzer. Organic carbon (OC) was eliminated from the bulk soil by combustion for 4 hours at 450º C (Cambardella et al., 2001) and the remaining inorganic carbon was measured. Total organic carbon (TOC) was then estimated as the difference between total carbon and inorganic carbon, after prior correction for weight loss.

2.3. Extraction of humic substances

The extraction of humic acids and fulvic acids from soils and sediments was performed according to the NAGOYA method (Kuwatsuka et al., 1992). Samples containing approximately 100 mg TOC were placed in 100 ml plastic bottles, and 60 ml of 0.1 M NaOH were added. After purging with N_2, the bottles were capped and shaken continuously for 24 h at 180 rpm, at room temperature. Then, 1.8 g of Na_2SO_4 was added as coagulant, and the suspension was centrifuged at 3000 rpm for 15 min. The supernatant was filtered through No. 6 filter paper, and the filter residue was washed twice with 60 ml of 0.1 M NaOH containing 1.8 g Na_2SO_4. The filtrate was centrifuged to obtain the sample extract. Humic acids and fulvic acids were separated by acidifying the extract to pH 1 with 6 N H_2SO_4 and allowing it to stand overnight. The precipitated humic acid fraction was separated from the fulvic acid fraction by centrifugation at 3000 rpm for 15 min. The residue was washed twice with 30 ml of 0.1 N H_2SO_4 and the washings were combined with the fulvic fraction. The humic acid fraction was then dissolved in 0.1N NaOH containing 30 g L^{-1} of Na_2SO_4 after neutralization with 0.2 N NaOH, and centrifuged at 3000 rpm for 20 min to remove impurities. The HA were re-precipitated by acidification to pH 1 and recovered by centrifugation.

2.4. UV-visible spectroscopy

Absorbance measurements were made in a 1 cm quartz UV-visible cell, in a Varian Cary 50-Bio UV-Visible Spectrophotometer, at room temperate (25± 3 ºC). The HA and FA were diluted with 0.1 N NaOH containing 30 g L^{-1} of Na_2SO_4 and the absorbances read at 400 nm (A_{400}) and at 600 nm (A_{600}). The absorbance of a blank solution was subtracted from those of the samples. Two spectrophotometric indices, i.e., Δlog K and RF, were then obtained in order to evaluate the UV-visible absorption characteristics of humic substances. The Δlog K index was calculated as Δlog K = log (A_{400}/A_{600}) (Kumada, 1987). For determination of the RF, the concentration of organic carbon (g L^{-1}) in the humic and fulvic acid solutions was determined by a colorimetric method with a potassium dichromate-sulfuric acid solution as reagent (Tatsukawa, 1966). The RF was then obtained as the absorbance at 600 nm per mg C mL^{-1} divided by a factor of 0.0648 (Ikeya and Watanabe, 2003).

2.5. Fluorescence spectroscopy

Fluorescence measurements were conducted with a Horiba Jovin YvonSPEX fluoromax-3 fluorometer at room temperature (25± 3 ºC). Both HA and FA fractions were diluted with each blank solutions and the pH adjusted to 8.

Analytical procedures for determining EEMs have been reported elsewhere (Maie et al., 2006; Yamashtia and Jaffé, 2008). For correction of synchronous spectra, inner-filter correction (McKnight et al., 2001) was carried out by use of absorbance spectra, and the EEMs of Milli-Q water were then subtracted from the sample EEMs. The specific instrument components were also corrected with excitation and emission correction files supplied by the manufacturers. Fluorescence intensities in EEMs were corrected to the peak area of the water Raman scatter (excitation = 350 nm) analyzed daily (Cory and McKnight, 2005), and then converted to quinine sulfate units (QSU) (Yamashita and Tanoue, 2003). The blank EEMs of HA and FA were corrected by use of the same procedures as for the samples, and then subtracted from corrected sample EEMs.

2.6. PARAFAC

The PARAFAC modeling for EEMs of natural organic mater has been described in detail elsewhere (Stedmon et al., 2003; Ohno and Bro, 2006). The range of wavelengths used for the PARAFAC model were 260-455 nm and 290-500 nm for excitation and emission, respectively. The analysis was carried out in MATLAB with the "N-way toolbox for MATLAB" (Andersson and Bro, 2000). Two to eight components were computed for all samples including both the FA and HA fractions (n = 56). Determination of the correct number of components was assessed by the core consistency diagnostic score (Bro and Kiers, 2003; Ohno and Bro, 2006).

2.7. Statistical analyses

One way analysis of variance (ANOVA) was applied to test differences among groups of samples. The statistical analyses were carried out with the SPSS statistical package (version 15.0, 2006). Principal Components Analysis (PCA) was performed with Statistica 8.0 software.

3.- Results and discussion

3.1. General characterization of soils and sediments

The general parameters analyzed for the studied soil and sediment profiles are shown in Table 1. The sediment profiles were slightly basic (pH 8.2-7.3) whereas the soils were neutral or acidic (pH 7.1-4.3). Interestingly, the most acidic conditions (pH 4.3) were observed in the reclaimed salt marsh at depth (55-70 cm), which may be due to oxidation of iron sulfides in this soil layer because of the drainage of the marsh (Van Breemen, 1982).

Table 1: Description of sampling sites, pH, total organic carbon (%), total nitrogen (%), total sulfur (%) and TOC/TN ratio of the studied soils and sediments.

	Site	Vegetation	Depth (cm)	pH	TOC (%)	TN (%)	TS (%)	TOC/TN
Urdaibai Esturay (43º 24' N 2º 41' W)	Tidal channel I (sediments)	bare sediments	0-5	8.2	1.4	0.13	0.70	10.8
			20-25	8.0	1.9	0.17	3.08	11.5
			40-50	7.9	1.0	0.12	0.95	8.4
	Tidal channel II (sediments)	bare sediments	0-5	7.6	1.2	0.11	0.24	10.9
			20-25	8.0	1.0	0.12	0.14	8.5
			50-60	7.3	0.9	0.09	0.08	9.9
	Natural salt marsh	*Halimione portulacoides*	0-5	6.4	6.7	0.49	0.28	13.6
			20-25	6.4	2.3	0.26	0.13	8.9
			45-55	6.5	1.2	0.14	0.15	8.9
	Regenerated salt marsh	*Halimione portulacoides*	0-5	6.7	3.1	0.30	0.19	10.3
			20-25	6.6	2.7	0.25	0.18	10.7
			40-50	6.8	1.6	0.18	0.13	8.7
	Polder (reclaimed salt marsh)	grassland	0-5	5.5	6.6	0.59	0.11	11.2
			20-25	7.1	1.2	0.15	0.04	8.4
			40-50	6.9	1.4	0.16	0.06	9.1
			55-70	4.3	1.3	0.13	0.70	10.1
Foz Estuary (43º 33' N 7º 15' W)	Tidal channel (sediments)	bare sediments	0-5	7.8	2.8	0.21	1.35	13.4
			20-25	7.8	2.8	0.29	0.99	9.5
			50-55	7.8	1.7	0.15	1.20	11.4
	Salt marsh I	*Halimione portulacoides*	0-5	4.8	15.0	0.97	0.96	15.4
			20-25	6.4	5.6	0.47	0.34	11.9
			45-50	7.1	1.8	0.18	0.32	10.4
	Salt marsh II	*Juncus maritimus*	0-5	6.2	21.3	1.45	0.63	14.7
			20-25	6.0	9.3	0.60	0.36	15.6
			40-50	6.2	4.6	0.38	1.37	12.2
	Abandoned polder	*Juncus maritimus*	0-5	6.2	14.1	1.02	0.45	13.9
			20-25	6.5	3.5	0.30	0.15	11.4
			40-50	6.5	0.9	0.10	0.09	8.9

Accumulation of TOC was greater in soils than in sediments, with the highest values at the surface layers and decreasing with depth in all the profiles (Table 1). Furthermore, the Foz soil profiles contained more TOC than the Urdaibai soil profiles, especially at the surface layers. This may be related to a higher tidal influence in the sampled soils of the Foz Estuary than in those of the Urdaibai Estuary. The longer period of flooding would lead to an oxygen poor geochemical environment, which implies a slower decomposition rate of the organic matter (D`Angelo and Ready, 1999) and therefore greater accumulation of TOC in the soil.

Total nitrogen (TN) was well correlated with TOC ($r = 0.99$, $N = 28$), indicating the organic origin of most of the nitrogen. As regard the TOC/TN ratio, the highest values were found at the surface. Furthermore, this ratio tended to decrease with depth in most of the studied profiles (Table 1). Several authors have reported a decrease in the TOC/TN ratio on litter and soil organic matter during the decomposition of marsh vegetation such as *Juncus sp.* and *Halimione sp.* (Newell et al., 1984;

Buth and de Wolf, 1985; Mclachlan and Vandermerwe, 1991; Kuehn et al., 2000; Chabbi and Rumpel, 2004).

The correlation between the sulfur content (TS) and the TOC content enables two groups of samples to be distinguished (Fig. 2). The first comprises the surface and subsurface soils, which present mainly oxic conditions and thus, most of the sulfur is organic and highly correlated with TOC (r = 0.85, n = 13). In this group of samples the TOC/TS was high (average value 25.7). However, in the other group of samples the TOC/TS ratio was much lower (average value 5.0) and TS was not significantly correlated with TOC (r = 0.46, n = 15). This second group of samples comprises the sediment profiles and the deepest layers of the soils where there is a predominance of anoxic conditions and the consequent formation and accumulation of inorganic sulfur as iron sulfides (Otero and Macías, 2002, 2003).

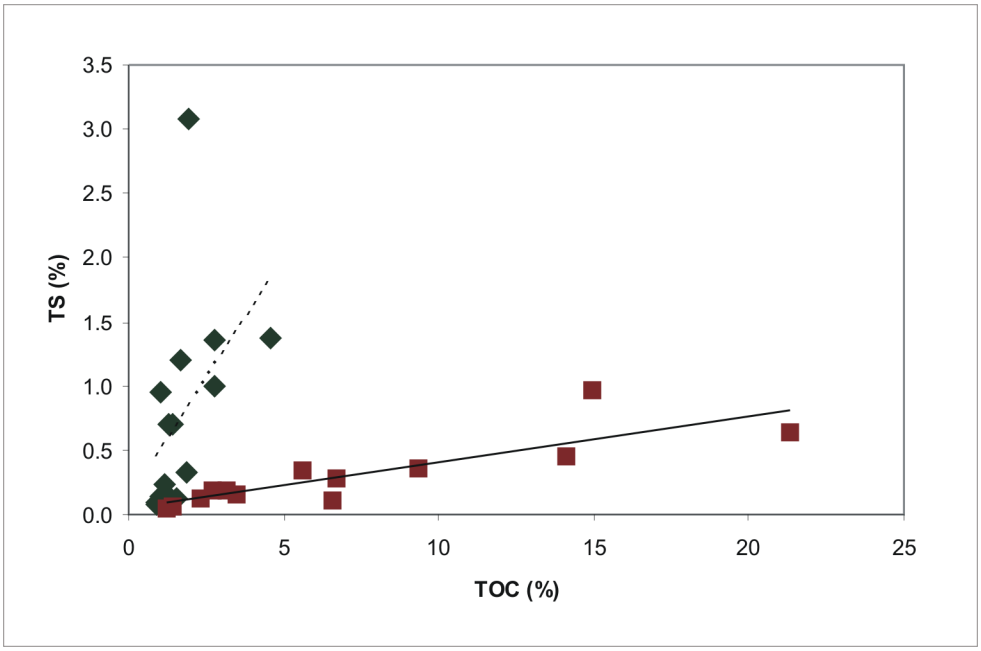

Figure 2. Plot of TOC (%) against TS (%) values for the studied samples. Two groups of samples are distinguished: squares: surface and subsurface layers of soils where the sulfur is predominantly organic; rhombus: sediments and deepest soil layers, where there is an accumulation of inorganic sulfur. Lines: least squares fit to each group of samples.

Table 2 shows the Δlog K and RF indexes, calculated for fulvic and humic acids from the UV-visible measurements. It is important to remember that the Δlog K value decreases with the development of

conjugation systems in the organic molecules, whereas the RF value is influenced by the proportion of non- or less colored moieties, and increases when the proportion of these non-colored molecules is lower (Ikeya and Watanabe, 2003). Thus, high RF values and low Δlog K values will be related to higher humification of humic substances and vice versa. The classification diagram for humic acids proposed by Kumada (1987) is shown in Figure 3. In this classification, HA are grouped into five main types: A, B, R_p, P_0 and P, according to their position on the graph of RF plotted against Δlog K. It can be observed that all of the HA under study were included in Type R_p, which indicates the immature character of the studied humic acids. Furthermore, in all the profiles the lowest RF values and highest Δlog K values were found at the surface layers (Table 2; Fig. 3), indicating a lower degree of humification of HA at surface. Since this classification was created exclusively for the HA, FA were not included in the diagram. However, it is interesting to note that all the FA presented much lower RF values and much higher Δlog K values than the HA (Table 2), which indicates the lower degree of humification of FA than of their HA counterparts.

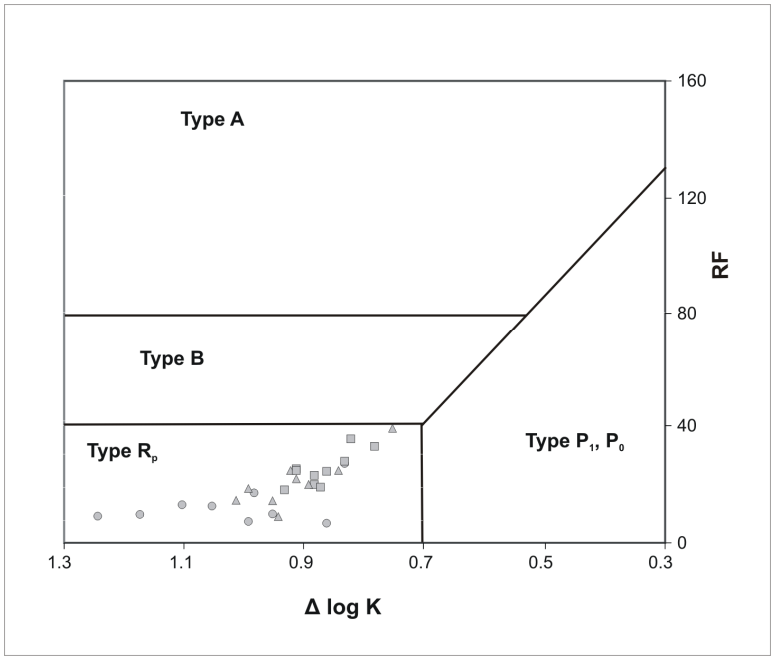

Figure 3. Classification of humic acids according to Kumada (1987). Humic acids from the studied soils and sediments profiles are represented as follows: circles (surfaces layers); triangles (subsurface layers) and squares (bottom layers).

Table 2. Two spectrophotometric indices, RF and Δlog K, calculated from absorbance measurements for fulvic and humic acids.

				Fulvic Acids		*Humic Acids*	
	Site	Vegetation	Depth (cm)	RF	Δlog K	RF	Δlog K
Urdaibai Esturay	Tidal channel I (sediments)	bare sediments	0-5	0.9	1.3	12.0	1.0
			20-25	1.7	1.2	25.0	0.8
			40-50	1.6	1.3	20.8	0.9
	Tidal channel II (sediments)	bare sediments	0-5	0.9	1.3	9.8	1.0
			20-25	2.7	1.2	37.8	0.8
			50-60	3.2	1.2	34.7	0.8
	Natural salt marsh	*Halimione portulacoides*	0-5	1.3	1.2	11.9	1.2
			20-25	1.2	1.2	22.6	0.9
			45-55	1.5	1.2	27.9	0.8
	Regenerated salt marsh	*Halimione portulacoides*	0-5	1.5	1.2	14.4	1.1
			20-25	2.0	1.1	20.9	0.9
			40-50	2.3	1.0	32.3	0.8
	Polder (reclaimed salt marsh)	grassland	0-5	2.6	1.0	27.2	0.8
			20-25	1.3	1.2	16.0	1.0
			40-50	1.9	1.3	23.7	0.9
			55-70	1.9	1.5	25.6	0.9
Foz Estuary	Tidal channel (sediments)	bare sediments	0-5	1.5	1.3	9.2	0.9
			20-25	1.0	1.3	11.2	0.9
			50-55	2.0	1.3	20.1	0.9
	Salt marsh I	*Halimione portulacoides*	0-5	0.5	1.4	11.3	1.2
			20-25	1.5	1.3	19.6	1.0
			45-50	1.5	1.4	25.1	0.9
	Salt marsh II	*Juncus maritimus*	0-5	1.9	1.2	14.9	1.1
			20-25	2.3	1.2	16.2	1.0
			40-50	2.1	1.3	19.3	0.9
	Abandoned polder	*Juncus maritimus*	0-5	1.8	1.3	18.4	1.0
			20-25	1.8	1.3	25.1	0.9
			40-50	1.2	1.4	24.9	0.9

3.2. Assignment of fluorescent components

The five components model was selected as the most suitable, since core consistency was 83.6 % and decreased dramatically after this number of components. The model explained 99.7 % of the variability in the dataset. The selection of the five component model does not mean that the EEMs only contained five different fluorophore components, but that these selected five components are the most representative since they are present in most of the samples, can be modeled and validated by the trilinear model and can explain most of the variation (Stedmon et al., 2003). The five components modeled are shown as EEM in Figure 4.

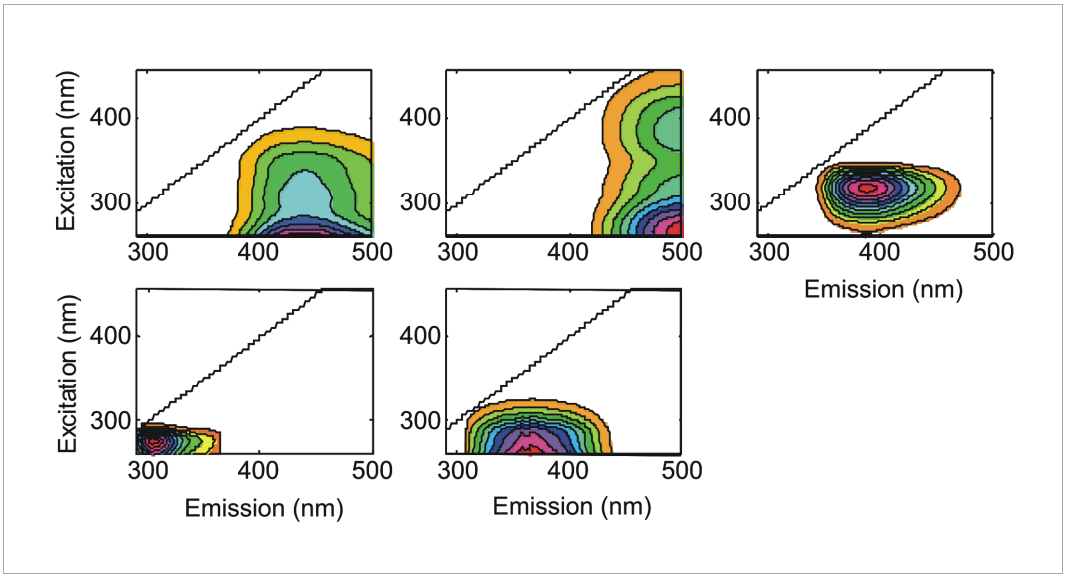

Figure 4. Contour plots of five fluorescent components obtained by PARAFAC.

The assignment of these five identified components is made according to previously identified fluorophores (Table 3). Component 1 (C1, Ex_{max} <260 (305), Em_{max} 439) is similar to previously reported humic-like fluorophores in DOM (Coble, 1996; Parlanti et al., 2000; Stedmon et al., 2003; Baker and Spencer, 2004; Banaitis et al., 2006; Holbrook et al., 2006; Cumberland and Baker, 2007; Hall and Kenny, 2007) as well as to terrestrial humic-like components obtained by PARAFAC (Cory and McKnight, 2005; Stedmon and Markager, 2005; Yamashita and Jaffé, 2008), and, in other studies, it has also been assigned to fulvic acid-like components (Chen et al., 2003; He et al., 2006). Component 2 (C2, Ex_{max} 265 (385), Em_{max} >500) has not been found in the traditional definition of DOM (Coble, 1996; Parlanti et al., 2000), but similar fluorophores were found in HS fractions, especially the HA fraction from different environments (Alberts and Takács, 2004a; Sierra et al., 2005a; He et al., 2006). Components similar to Component 2 have also been found in DOM in aquatic environments (Stedmon and Markager, 2005; Cory and McKnight, 2005; Yamashita et al., 2008; Yamashtia and Jaffé, 2008). Component 2 also appears to be a humic-like component, attributed to fluorophores of high molecular weight (Stedmon et al., 2003). Component 3 (C3, Ex_{max} 320, Em_{max} 388) is comparable to marine humic like-compounds traditionally defined in DOM (Coble et al., 1966; Parlanti et al.,

2000), and also to microbial-origin PARAFAC components (Cory and McKnight, 2005; Yamashita et al., 2008; Yamashtia and Jaffé, 2008). Component 4 (C4, Ex_{max} 275, Em_{max} 304) is classified as a tyrosine-like, protein-like component (Coble et al., 1996; Parlanti et al., 2000; Sierra et al., 2005a; Banaitis et al., 2006; Onho and Bro, 2006). Component 5 (C5, Ex_{max} <260, Em_{max} 365) was assigned to a tryptophan-like fluorophore (Coble et al., 1996; Parlanti et al., 2000; Stedmon et al, 2003; Baker and Spencer, 2004; Banaitis et al., 2006). However, the fluorescent characteristics of C5 were also similar to those of gallic acid, a major constituent of tannins (Fukushima et al., 1997; Maie et al., 2007), but were not similar to those of tannins (Maie et al., 2007). In the present study, C5 is assigned to a tryptophan-like component because it is unlikely that a monomer of gallic acid occurred in HS. In addition, PCA analysis in the present study showed that C4 and C5 were clustered similarly (as mentioned below), suggesting that sources and transformations of C4 and C5 in HS were similar.

Thus, from their fluorescent characteristics, C1, C2, C3, C4, and C5 found in HA and FA fractions were characterized as fulvic acid-like, humic acid-like, microbial humic-like, tyrosine-like, and tryptophan-like components, respectively (Table 3).

The 5 components determined in the present study were similar to PARAFAC components found in DOM in soils and in water soluble fractions from soils (Table 3: Ohno and Bro, 2006; Ohno et al., 2007; Hunt et al., 2008; Fellman et al., 2008) and DOM in a wide range of aquatic environments (Table 3: Stedmon and Markager, 2005; Cory and McKnight, 2005; Yamashita and Jaffé, 2008). Comparison of fluorescence characteristics in HS obtained in the present study and in DOM in surface waters from Florida coastal Everglades (Yamashita and Jaffé, 2008) and tropical rainforest rivers (Yamashita et al., in prep.) is illustrated in Fig. 5.

Three humic-like fluorescent components from HS, i.e., fulvic acid-like (C1), humic acid-like (C2), and microbial humic-like components (C3), were similar to fluorescent components found in surface water DOM (Fig. 5), strongly suggesting that fluorophores comprised of HS may move to soil interstitial water and thus be transferred to DOM in aquatic

environments without significant changes in optical characteristics of fluorophores. In agreement with this, previous studies have reported salt marshes as major sources of DOM to coastal waters (Moran and Hodson, 1994; Clark et al., 2008).

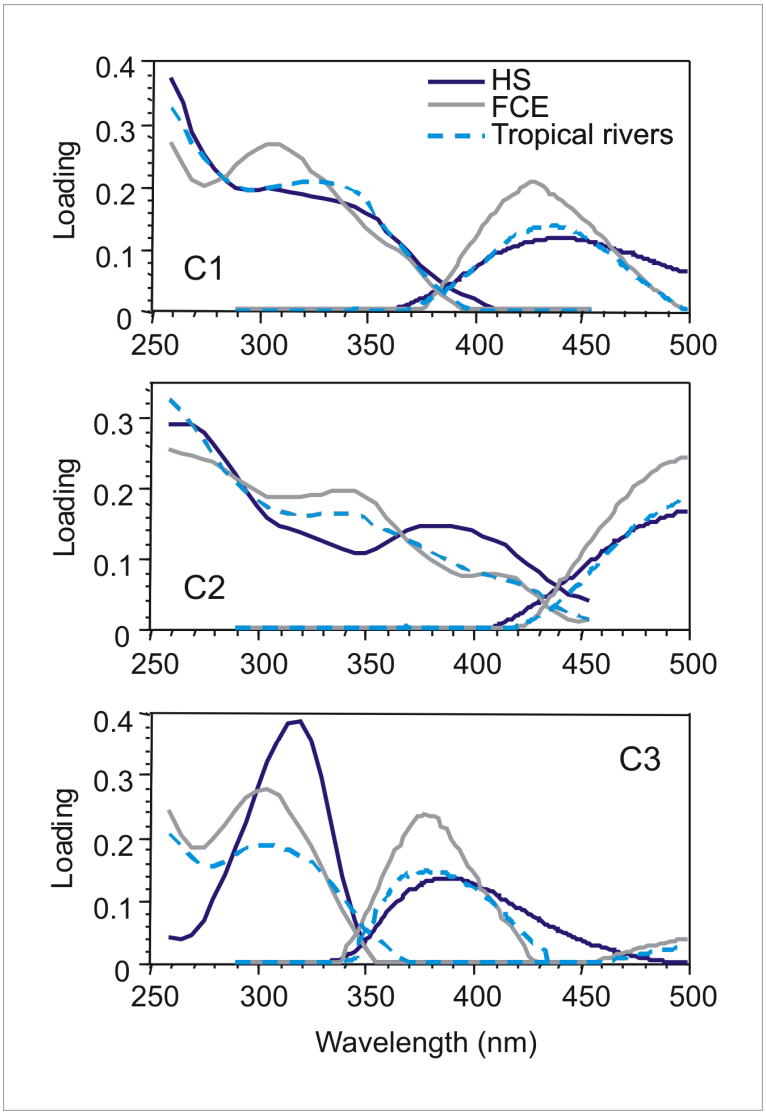

Figure 5. Comparison, by PARAFAC, of excitation and emission spectra of fluorescent components of humic substances in the present study (HS), DOM from Florida Coastal Everglades (FCE; Yamashita and Jaffé, 2008), and DOM from tropical rivers (Yamashita et al., in prep.). C1) Fulvic acid-like component; C2) Humic acid-like component; C3) Microbial humic-like component.

Table 3: EEM fluorescence peaks identified in previous studies.

Reference	OM Source	Peak label	EEM Fluorescence Ex_{max} (nm)	Em_{max} (nm)	Assigned compounds
Coble, 1996	DOM	B	275	310	Tyrosine-like, protein-like
		T	275	340	Tryptophan-like, protein-like
		A	260	380-460	Humic-like
		M	312	380-420	Marine humic-like
		C	350	420-480	Humic-like
Parlanti et al., 2000	DOM	α	330-350	420-480	Humic-like
		ά	250-260	380-480	Humic-like
		β	310-320	380-420	Marine humic-like
		γ	270-280	300-320	Tyrosine-like, protein-like
		δ	270-280	320-350	Tryptophan-like, protein-like
Chen et al., 2003	DOM	Region I	<250	<350	Simple aromatic proteins
		Region II	<250	<350	Simple aromatic proteins
		Region III	<250	>350	Fulvic-like
		Region IV	250-280	<380	Soluble microbial by-products
		Region V	>280	>380	Humic-like
Alberts&Takács, 2004	NOM and IHSS HS	1	224-264	428-458	Humic-like
		2	321-459	433-513	Humic-like
		***3	223-355	446-468	Highly conjugated systems
		***4	227-379	465-505	Highly conjugated systems
Alberts et al., 2004	NOM	-	340	445	Aquatic humic-like
		-	230	430	Aquatic humic-like
		-	320	424	Marine humic-like
		-	300	350	Tryptophan-like, protein-like
Baker&Inverarity, 2004	Freshwater DOM	-	280	350	Tryptophan-like, protein-like
		-	220	350	Tryptophan-like, protein-like
Baker&Spencer, 2004	DOM	T220	220	350	Tryptophan-like, protein-like
		T280	280	350	Tryptophan-like, protein-like
		B	220	305	Tyrosine-like, protein-like
		A	220-250	400-480	Humic-like
		C	300-380	400-480	Fulvic-like
Sierra et al., 2005a	HS	ά	260*, 265**	460*, 525**	Humic-like
		α	310*, 360**	440*,520**	Humic-like
		***β	320	425	Marine humic-like
		***δ/γ	270-275	305-320*, 330-345**	Protein-derived and phenolic
Cumberland & Baker, 2007	Freshwater DOM	H-L	220-250	400-460	Humic-like
		F-L	300-340	400-460	Fulvic-like
Clark et al., 2008	Coastal DOM and salt marsh plant leachates[¶]	B	271-275	301-310	Tyrosine-like, protein-like
		T	275	333-340	Tryptophan-like, protein-like
		A	260-274	380-460	Humic-like
		M	306-312	380-423	Marine humic-like
		C	343-350	420-480	Terrestrial humic-like

* Max. for fulvic acids. **Max. for humic acids. *** Only found in humic acids. [¶] Data of leachates postirradiation not inlcuded.

Table 3 (continuation): EEM-PARAFAC model components identified in previous studies. Secondary excitation maxima are shown in brackets.

		EMM-PARAFAC models			
Reference	OM Source	Component	Ex_{max} (nm)	Em_{max} (nm)	Assigned compounds
Stedmon et al., 2003	Estuarine DOM	1	<240	436	Humic-like (terrestrial origin)
		2	<240	416	Humic-like(terrestrial origin)
		3	270(360)	478	HMW humic-like (terrestrial origini)
		4	325(250)	416	humic-like (terestrial origin)
		5	280(<240)	368	Protein-like (authoctonous)
Stedmon & Markager, 2005	Freshwater, wastewater and estuarine DOM	1	<250	448	Humic-like
		2	<250(305)	412	Humic-like
		3	<250(385)	504	Fulvic-like
		4	<250(360)	440	Fulvic-like
		5	325	448	Humic-like
		6	<250(320)	400	Humic-like
		7	280	344	Tryptophan-like, protein-like
		8	275	304	Tyrosine-like, protein like
Banaitis et al., 2006	Soil and vegetation DOM	1	275	320	Tyrosine-like, protein like
		2	<240(280)	340	Tryptophan-like, protein-like
		3	<240(275,330)	450	Humic substances-like
		4	(270)310	440	Humic substances-like
		5	<240(30)	400	Humic substances-like
He et al., 2006	IHSS HS	1	<240(318)	438	Fulvic-like
		2	252	>498	Humic-like
		3	<240	390	Fulvic-like
Holbrook et al., 2006	Freshwater DOM	1	240(350)	456	Humic-like
		2	240(305)	396	Fulvic-like
		3	<230(280)	340	Protein-like
Ohno&Bro, 2006	DOM	1	(275)350	450	Humic and fulvic-like
		2	<250	450	Humic and fulvic-like
		3	<250(320)	400	Humic and fulvic-like
		4	275	300	Protein-like
		5	275	320	Protein-like
Hall&Kenny, 2007	Estuarine DOM	1	240(310)	420	Humic-like
		2	240-260(370)	480	Humic-like
		3	220-240(330)	400	Marine humic-like
		4	220-240(280)	>550	Aminoacids
		5	<220(280)	400	Unassigned[+]
Onho et al, 2007	Soil DOM	1	339	470	Humic-like
		2	324	418	Fulvic-like
		3	<240	465	Humic substances-like
Yamashita et al, 2008	Estuarine DOM	1	<260	458	Terrestrial humic-like
		2	345	433	Terrestrial humic-like
		3	390(275)	479	Terrestrial humic-like
		4	280	318	Tryptophan-like, potein-like
		5	285	362	Non humic-like
		6	325(<260)	385	Marine humic-like
		7	270	299	Tyrosine-like, protein like
Yamashita and Jaffé, 2008	Terrestrial and Estuarine DOM	1	305(<260)	428	Terrestrial humic-like
		2	<260 (340, 405)	>500	Terrestrial humic-like
		3	<260	448	Terrestrial humic-like
		4	305 (<260)	378	Microbial humic-like
		5	265 (380)	462	Microbial humic-like
		6	345	424	Terrestrial humic-like
		7	295	340	Protein-like
		8	275	324	Protein-like
This study	Soil and sediment HS	C1	<260(305)	439	Fulvic-like
		C2	265(385)	>500	Humic-like
		C3	320	388	Microbial origin
		C4	275	304	Tyrosine-like, protein like
		C5	<260	365	Tryptophan-like, protein-like

[+]Attributed to data scatter or contamination.

3.3. Distribution of fluorescent components in humic and fulvic acids

Figure 6 shows the average contribution of the 5 PARAFAC components for HA and FA. The contribution of the 5 components in all HA and FA fractions are also summarized in Table 4 Interestingly, fulvic acid-like (C1), humic acid-like (C2), microbial humic-like (C3), and tryptophan-like (C5) components were found in all of the samples, irrespective of differences in HA and FA fractions (Table 4). He et al (2006) and Ohno and Bro (2006) also found that all 3 PARAFAC components were shared in all of their HS samples, i.e., 4 IHSS HA and 2 IHSS FA samples, and indicated the consistency with the new paradigm for the structure of soil humic substances (Sutton and Sposito, 2005). In accordance, the present EEM-PARAFAC result of HS obtained from estuarine sediments and soils also support the new paradigm, i.e., that soil/sediments HA and FA are composed of relatively low molecular weight components that interact strongly each other by hydrophobic interactions and hydrogen bonds, and they cannot be separated effectively by chemical or physical methods (Sutton and Sposito, 2005).

There were statistically significant differences between the contributions of each of the 5 components to HA and FA groups (ANOVA, $p < 0.05$). The C1 component was characterized as a fulvic acid-like component from its fluorescence characteristics (Table 3). In accordance with its fluorescence characteristics, C1 was the dominant fluorescence component in the FA (Fig. 6). In contrast, the predominant component in the HA group was the C2 (Fig. 6) which was characterized as a humic acid-like component by its fluorescence characteristics (Table 3). It should be noted that contributions of fulvic acid-like component (C1) were higher in FA fraction than in the corresponding HA fraction in almost of all the samples (Table 4) and those of the humic acid-like component (C2) were always higher in HA fraction than in corresponding FA fraction (Table 4).

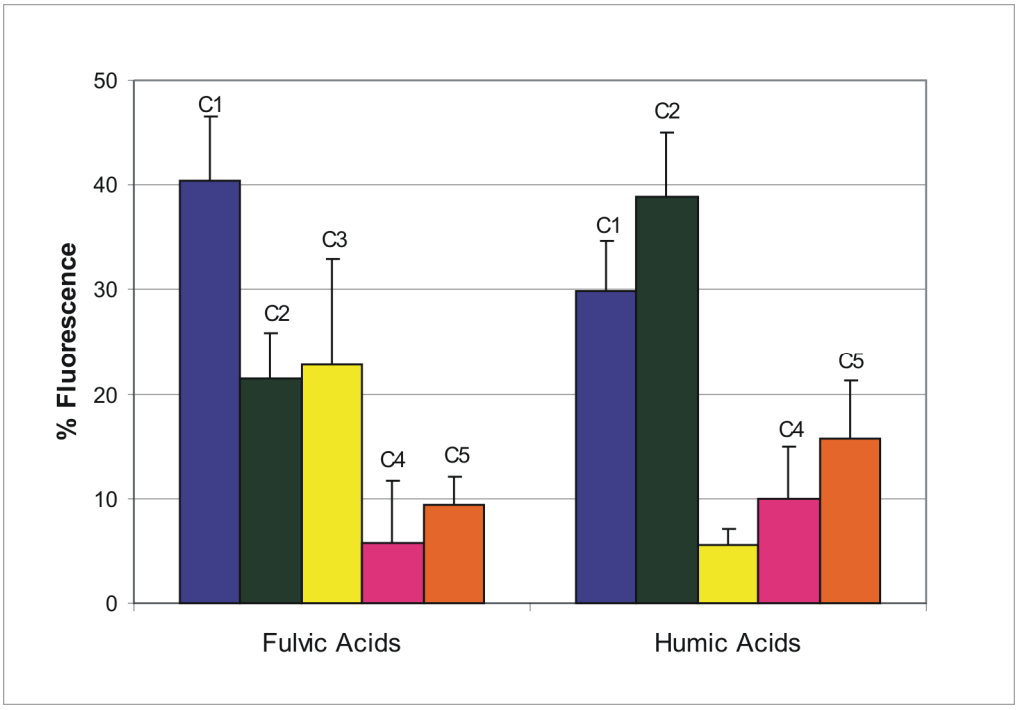

Figure 6. Average relative contributions (%) of each of the five PARAFAC components for fulvic and humic acid fractions. Error bars (standard errors) are bidirectional, with lower limits omitted for clarity.

Such compositional differences in fluorescent groups between HA and FA fractions (Fig. 6) are consistent with those found in previous EEM studies, i.e., a red shift in fluorescence maxima in the HA fraction compared with the FA fraction (Mobed et al., 1995; Alberts and Takacs, 2004a; Sierra et al., 2005a). A red shift in the fluorescence maxima has been considered to be due to the presence of high molecular weight fractions, electron-withdrawing substitutes, and a higher degree of conjugation (Mobed et al., 1996). Thus, significant differences in the contributions of C1 and C2 between FA and HA fractions were also consistent with differences in molecular weight distribution between HA and FA fractions (Sutton and Sposito, 2005).

The C3, characterized as a microbial humic-like component (Table 3), is much more abundant in FA than in HA (Fig. 6). It presents the highest variability among the studied group of FA samples (Fig. 6). The C3 component was the least abundant in the HA fraction (Fig. 6) and presented a relatively narrow range in all the HA (Table 4). As regards the variability in the composition of fulvic acids, 78.6 % of them (n = 22/28,

Table 4) were predomianted by C1, and the remaining 21.4 % of the samples (n =6/28), were predominated by C3. The previous EEM studies of soil/sediment HS, however, have not shown different peaks of microbial humic-like component (Sierra et al., 2005a; Alberts and Takács, 2004a, Mobed et al., 1996). Sierra et al (2005a) observed the shoulder of microbial humic-like component in soil/sediment HA fractions but not in their FA fractions and indicated that the absence of the microbial humic-like component in FA fractions may be due to 1) solubility characteristics and 2) overlapping the intense fulvic acid-like fluorophore in FA fraction.

In marine environments where the contribution of terrestrial humic substances is small, a marine (microbial) humic-like fluorophore, similar to C3, is known to predominate in EEM (Coble e al., 1998; Del Castillo et al., 1999; Yamashita and Tanoue, 2003; Maie et al., 2006) and to be a major PARAFAC component (Murphy et al., 2008; Yamashita et al., 2008). EEM-PARAFAC in the present study separated the microbial humic-like components from fulvic acid-like components and indicated that microbial humic-like components are rich in FA fraction, but not in HA fractions. This evidence strongly indicates that peaks found in EEM of FA fractions were composed of a mixture of microbial humic-like and fulvic acid-like components. In addition, the differences in contribution of the microbial humic-like component (C3) in the FA and HA fractions provided insights for source characteristics of HS, i.e., stronger microbial sources in the FA fraction than in the HA fraction.

In the protein-like components, the tryptophan-like component (C5) was evident in all FA and HA. On the other hand, the tyrosine-like component (C4) was not found in 5 of the 28 FA (Table 4). The protein-like fluorophores were also found in EEM of relatively poorly humified sediment/soil FA and HA (Sierra et al., 2005a). Thus, evidence of protein-like components in FA and HA fractions would be due to the low humification of the studied samples (Fig. 3). This is consistent with the findings in similar coastal environments, of a predominance of labile and low decomposed organic components in the composition of HA and FA (Chapters III and IV of this dissertation; Santín et al., 2008a, b). Interestingly, both protein-like components were generally more abundant

in HA than in FA (Table 4; Fig. 6). This result differs from that obtained by Sierra et al. (2005a) who reported better visualization of the protein signal in the EEM from FA than in the EEM from HA. On the other hand, Sierra et al (2005b) studied HS from subtropical coastal environments and observed a higher nitrogen content in HA than in FA, most of which was attributed to proteinaceous materials. Furthermore, the tryptophan-like fluorescence contribution is generally greater than the tyrosine-like contribution in both FA and HA fractions (Table 4; Fig. 6). This may be indicative of a low decomposition of the proteinaceous materials, since tryptophan-like fluorescence appears to be indicative of less degraded peptide material and tyrosine-like fluorescence of more degraded peptide material (Yamashita and Tanoue, 2003, 2004). This suggests that labile protein-like components occurred in FA and HA fractions by interactions with other organic molecules and were protected from microbial degradation (Sutton and Sposito, 2005), and that such interactions were strongly evident in HA compared to FA in the studied soils and sediments.

Table 4: Relative concentrations (%) of the five identified fluorophores in the PARAFAC model for all the studied samples.

			Fulvic Acids					Humic Acids				
	Site	Depth (cm)	C1 (%)	C2 (%)	C3 (%)	C4 (%)	C5 (%)	C1 (%)	C2 (%)	C3 (%)	C4 (%)	C5 (%)
Urdaibai Esturay	Tidal channel I (sediments)	0-5	47	20	16	6	12	26	30	5	14	26
		20-25	44	21	16	7	12	31	41	10	7	11
		40-50	38	24	30	1	7	29	41	6	10	15
	Tidal channel II (sediments)	0-5	31	18	36	4	10	23	27	4	18	29
		20-25	46	28	19	1	6	29	39	6	12	14
		50-60	46	29	19	0	6	31	45	5	6	13
	Natural salt marsh	0-5	34	15	39	5	7	31	50	5	4	10
		20-25	35	19	39	0	7	34	39	5	8	14
		45-55	43	27	23	0	7	32	42	5	8	14
	Regenerated salt marsh	0-5	31	18	40	2	8	28	35	6	12	18
		20-25	39	21	28	5	7	30	36	6	10	18
		40-50	41	22	28	2	8	31	41	6	10	13
	Polder (reclaimed salt marsh)	0-5	45	18	22	6	9	35	34	7	10	14
		20-25	43	23	26	0	8	37	42	5	4	12
		40-50	44	25	8	8	15	35	45	5	4	11
		55-70	50	29	13	0	8	37	46	6	2	9
Foz Estuary	Tidal channel (sediments)	0-5	36	23	15	13	13	20	35	6	18	22
		20-25	27	16	38	9	9	18	24	3	22	32
		50-55	40	21	21	9	8	24	34	5	17	20
	Salt marsh I	0-5	37	15	16	18	13	26	44	4	12	13
		20-25	37	15	13	26	9	27	36	6	14	17
		45-50	43	25	23	3	6	32	46	4	6	11
	Salt marsh II	0-5	31	16	37	3	11	33	37	9	7	14
		20-25	45	21	17	7	10	30	37	7	11	14
		40-50	36	22	25	10	6	27	34	7	15	17
	Abandoned polder	0-5	48	16	14	8	14	34	36	7	9	13
		20-25	47	23	10	7	13	34	41	4	8	13
		40-50	47	29	9	4	12	34	50	4	2	10

3.4. Distribution of variability in fluorescence among samples

Although the greatest differences were between HA and FA groups (Table 4, Fig. 6), spatial and vertical variability in the contribution of each of the fluorophores were not clear (Table 4). Such results may be due to variable sources of organic matter in the study area. To clarify the similarity/dissimilarity in entire fluorophore compositions, PCA was carried out on the data corresponding to the relative abundance of each component (Fig. 7).

As expected from large differences in fluorophore compositions between HA and FA fractions (Fig. 6), the plots of HA and FA were distinguishable (Fig. 7b; ANOVA, $p < 0.01$ for principle component scores 1 and 2). Such differences were the result of differences in contribution of C1, C2, and C3 between FA and HA fractions (Figs. 6 and 7a). Moreover, PCA highlights the degree of sample heterogeneity. The scatter plots for both principle components in FA fraction indicated that variability in the fluorophore composition of FA was due to variation in the 5 components

(Fig. 7b). On the other hand, the plots in HA fraction were linearly distributed (Fig. 7b), indicating that the composition of HA was basically controlled by two end-members, namely, protein-components and the humic acid-like component (Fig. 7a).

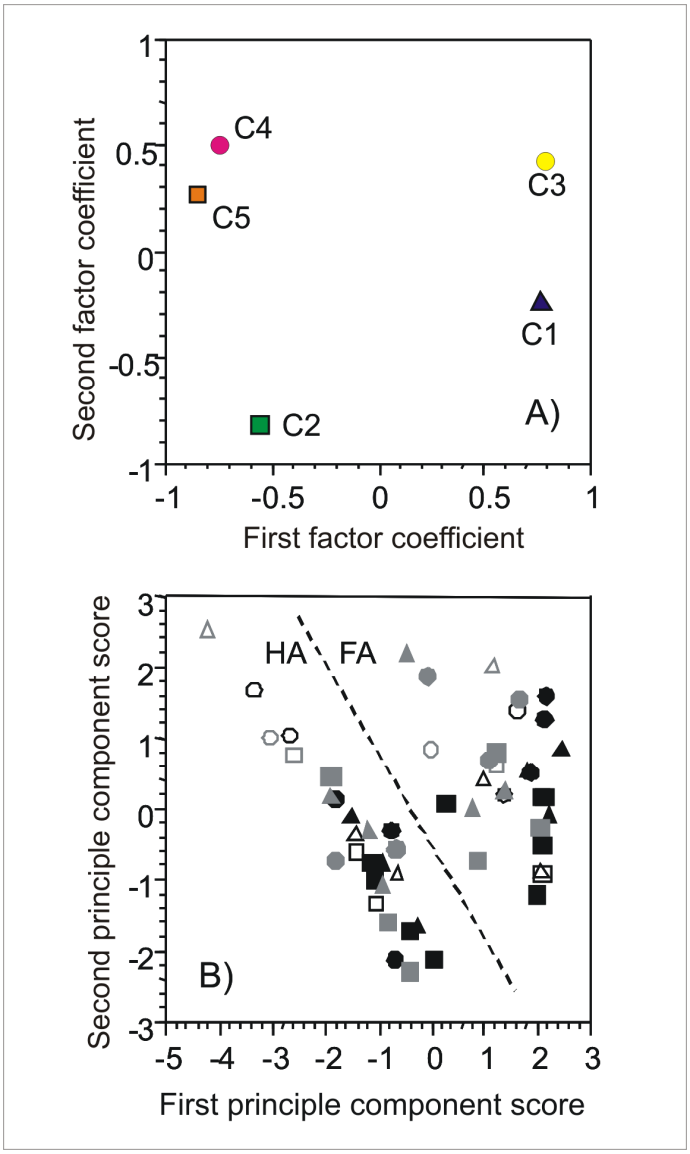

Figure 7. Results of principal component analysis (PCA) of the five PARAFAC components. A) Property-property plots between first and second factor coefficients; B) Property-property plots between first and second principle component scores. The dotted line was arbitrarily described. The first two principal components explained 57% and 26 % of the variance, respectively. Black and grey symbols indicate samples from the Urdaibai and Foz estuaries, respectively. Open and closed symbols indicate sediment and soil samples, respectively. Circles, triangles, and squares indicate samples from surface, subsurface, and bottom, respectively.

The PCA also revealed the differences in fluorophore composition of HS obtained between two estuaries, between sediments and soils, and among depths. The plots for FA distinguished between surface and bottom samples by the principle component score 2 (Fig. 7b; ANOVA, $p < 0.01$), although differences were not significant between surface and mid-depth samples or between mid-depth and bottom samples (ANOVA, $p > 0.05$). On the other hand, no significant differences were found among depths for the HA fractions (Fig. 7b; ANOVA, $p > 0.05$). The significant differences in principle component score 2 in FA fractions were due to different contributions by microbial humic-/protein-like components and fulvic acid-/humic acid-like components (Fig. 7b), suggesting compositional changes during humification in the FA fraction. A decrease in the contribution of protein-like fluorophores with depth was also found in FA extracted from sediment from mangrove dominated site (Sierra et al., 2005a), suggesting that such compositional changes in FA fraction during humification processes are likely to be ubiquitous in the environment.

As regards the FA fraction, the plots corresponding to the Urdaibai estuary showed more negative values for the principle component score 1 than those corresponding to the Foz estuary (Fig. 7b; ANOVA, $p < 0.01$). Such results were due to greater richness in protein-like components, especially tyrosine-like components (poor in fulvic acid- and microbial humic-like components), in the Foz FA than in the Urdaibai FA (Table 4). Differences between two estuaries were, however, not significant for the HA fraction (Fig. 7b; ANOVA, $p > 0.05$). The compositional difference in FA between estuaries may be the result of a poor degradation of OM and/or a greater marine influence in the Foz estuary than in the Urdaibai estuary and is consistent with differences in TOC contents between two estuaries (see section 3.1).

Significant differences between sediment and soil samples, were found in the principle component scores 1 and 2 in the HA fraction (Fig. 7b; ANOVA, $p < 0.05$). Such differences in HA fraction were the result of a large contribution of protein-like components in sediment samples compared with those in soil samples (Fig. 7). Such a large contribution of protein components observed in the tidal channel sediments may be the

result of marine inputs rich in proteinaceous materials and to the contribution of algaenan. Algaenan is a typical refractory component in algae, a highly paraffinic biopolymer which also contains proteinaceous materials in its structure (Knicker 2004). The mechanism whereby peptides in algaenan are protected against microbial degradation has been explained by the encapsulation pathway, that is, bio-labile proteinaceous material covalently bound to recalcitrant biopolymers (Knicker and Hatcher, 1997 and 2001). This view is consistent with the new paradigm of HS (Sutton and Sposito, 2005) as mentioned above. The non-significant differences in HA composition between depths and between two estuaries suggest that algaenan may be distributed randomly at depths and between the two estuaries. It should be noted that differences in PCA between sediment and soils samples were also not significant in the FA fraction (Fig. 7b; ANOVA, $p > 0.05$). Further studies, are necessary to clarify the factors controlling algaenan distribution in estuarine environments.

4.- Conclusions

The HA and FA extracted from estuarine sediments and soils were characterized by EEM fluorescence-PARAFAC in the present study. As a result, significant compositional differences between HA and FA were found. The representative fluorescent components in FA and HA fractions, i.e., C1 and C2, were defined as fulvic-acid like and humic acid-like components, respectively. In addition, the fluorescent characteristics of these components were similar to DOM in water soluble fraction from soil and DOM in surface water. Such similarities strongly indicate that these components will be useful for source characterization of DOM in wide range of aquatic environments. Even though all of the HS used for the present study were poorly humified HS, the results of PCA with EEM-PARAFAC components revealed significant differences between FA and HA, between sediments and soils, and among depths, indicating that EEM-PARAFAC is a reliable technique for characterization of HS in natural environments.

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Conclusiones

Conclusiones

En la presente Memoria de Tesis Doctoral se estudiaron suelos y sedimentos de varios sistemas estuarinos localizados en el litoral cántabro-atlántico español: los estuarios de Urdaibai (País Vasco), Villaviciosa (Asturias) y Foz (Galicia), la ría de Ortigueira (Galicia) y la laguna costera de Caldebarcos (Galicia).

La caracterización de los suelos y sedimentos se realizó combinando los métodos físico-químicos tradicionales utilizados en los estudios edafológicos con una amplia gama de técnicas espectroscópicas para la caracterización de la materia orgánica (MO): espectroscopía de luz UV-visible, espectroscopía de infrarrojo por transformada de Fourier, espectroscopía de fluorescencia, espectrometría de masas de isótopos estables, resonancia magnética nuclear de ^{13}C en estado sólido y pirólisis analítica. Los resultados obtenidos permiten extraer las siguientes conclusiones:

1. Las características de la MO en suelos y sedimentos de ambientes estuarinos reflejan la gran variabilidad propia de estos sistemas transicionales entre los ecosistemas terrestres y acuáticos. Pese a que la MO estudiada en los distintos sustratos muestra una alta variabilidad, comparte también, de modo general, determinadas características como son: bajo grado de humificación, baja proporción de compuestos aromáticos y una contribución elevada de compuestos tipo polisacárido y proteína.

2. El aumento del grado de humificación y/o descomposición de la MO estudiada está relacionada, en la mayoría de los casos, con un aumento del grado de aromaticidad y un aumento en la contribución de compuestos alifáticos no sustituidos y con una disminución de compuestos más fácilmente biodegradables (proteínas y polisacáridos).

3. En los suelos estuarinos se ha observado que, además de la vegetación, existen otros factores ambientales que influyen en las características de la materia orgánica edáfica, y deben ser, por tanto, apropiadamente considerados. Así,

a. Las sustancias húmicas caracterizadas en suelos de juncal (*Juncus maritimus*) de Caldebarcos y Villaviciosa muestran diferencias en su composición a pesar de estar bajo la influencia de la misma vegetación. Estas diferencias se han interpretado en función de los diferentes ambientes geoquímicos de las zonas muestreadas, de manera que, las condiciones óxicas, asociadas a texturas arenosas, favorecen el aumento de la humificación, mientras que condiciones subóxicas o anóxicas, relacionadas con texturas finas, favorecen la preservación de la MO.

b. Las sustancias húmicas de los suelos estuarinos colonizados por *Spartina maritima* en Ortigueira y Villaviciosa muestran un bajo grado de aromaticidad, alto contenido en nitrógeno y gran proporción de componentes alifáticos, lo que puede relacionarse con contribuciones orgánicas distintas a materiales vegetales, tales como algas, fitoplancton o microorganismos. En estos suelos, altamente afectados por la dinámica mareal, la posición fisiográfica dentro del ambiente estuarino es de suma importancia ya que condiciona los procesos de intercambio con los sistemas adyacentes y, por tanto, las características de la materia orgánica edáfica.

4. En la costa cantábrica de España los procesos de reclamado son un importante factor de perturbación en los sistemas estuarinos. En líneas generales, este reclamado lleva asociado un cese o disminución del grado de inundación y una maduración de los suelos. Como consecuencia, se favorece el asentamiento de vegetación no halófila, lo cual provoca profundos cambios en las

características de la materia orgánica edáfica. La dirección de estos cambios está condicionada por multitud de factores, entre ellos la condición original del sistema:

   a. En Villaviciosa, el reclamado permite el asentamiento de vegetación en lo que previamente era una llanura intermareal desnuda, aumentando la acumulación de carbono orgánico edáfico y permitiendo la acumulación de compuestos con mayor grado de humificación.
   b. En Urdaibai, el reclamado y la sustitución de la vegetación de marisma por pastizal disminuye el carbono acumulado en el suelo, aumenta la contribución de polisacáridos a la MO edáfica y reduce la proporción de compuestos derivados de lignina.

5. La marisma regenerada estudiada en el Estuario de Urdaibai presenta la misma vegetación y parámetros edáficos similares a la marisma natural adyacente. Sin embargo, se observa una menor acumulación de MO y una menor proporción de compuestos aromáticos derivados de lignina y contribuciones microbianas en comparación con la marisma natural. Considerando la MO como un indicador de la recuperación de marismas, los resultados ponen de manifiesto el largo tiempo necesario para el restablecimiento de sus características originales.

6. Por primera vez, se ha utilizado la combinación de espectroscopía de fluorescencia en tres dimensiones y análisis factorial paralelo para la caracterización de sustancias húmicas de suelos y sedimentos. Los cinco fluoróforos identificados se encontraron tanto en los ácidos húmicos como en los fúlvicos, lo que corrobora el nuevo paradigma de las sustancias húmicas como asociaciones supramoleculares. De estos cinco fluoróforos, el descrito como “tipo húmico” es el predominante en los ácidos húmicos, mientras que el “tipo fúlvico” lo es en los ácidos fúlvicos. Los otros tres fluoróforos

(el de origen microbiano, el “tipo tirosina” y el “tipo triptófano”) muestran también distribuciones diferentes en ambas fracciones de sustancias húmicas.