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Unraveling bacterioplanktonic ecology of the estuaries of Urdaibai and Bilbao in the Bay of Biscay by high-throughput sequencing

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Abstract

Planktonic communities are widely used as indicators of water pollution level, as they are vulnerable to water quality status. Indeed, it has been proposed that estuaries microbial composition changes during eutrophication period due to the increase of pollutants and hypoxia. In the present study, we characterized the bacterial community structure of two macro-mesotidal estuaries by sequencing the V4 region of the 16s rDNA gene. While Bilbao estuary crosses a densely populated urban area, the Urdaibai estuary is an UNESCO biosphere reserve.

In the case of Udaibai, some of the taxa characterizing that water mass community were found to be related either with an anthropogenic (Enterobacteriaceae, Clostridium, Ruminococcus, Thiothrix, etc.) or terrestrial origin (Comamonadaceae, Rhodocyclaceae, etc.). Expectedly, the downstream outer waters bacterial community was characterized by OTUs (Operational Taxonomic Units) mainly related to the oceanic realm.

In the estuary of Bilbao, ont he one hand, an increase in freshwater bacteria (*Comamonadaceae* and *Sphingobacteriaceae*) was observed in high precipitation periods compared to the predominately marine-like bacteria (*Rhodobacterales* and *Oceanospirillales*) that were found in low precipitation periods. Notably, we observed a significantly higher relative abundance of *Comamonadaceae* than previously described in other estuaries, for this reason the gene transcription abundances changes of these bacteria were measured along the different water masses of the estuary identifying the differential expression of diverse functional pathways. For instance, the activation of cellular movement and membrane transport proteins were evidenced in stagnant eutrophic waters for *Limnohabitans* bacteria. Futhermore, in summer, a low dissolved oxygen (DO) concentration, high temperature, and high chlorophyll concentration period in the inner euhaline water (samples with salinity >30 ppt). Those samples were characterized by a high

abundance of facultative anaerobes (*Cryomorphaceae* and *Candidatus Aquiluna rubra*) and with the over-expression of anaplerotic and anaerobic metabolic pathways. Additionally, microorganisms related to biological treatment of wastewater (e.g Bdellovibrio and Zoogloea) were detected in the samples immediately downstream of the Bilbao Wastewater Treatment Plant (WWTP). However their metabolic activity and presence are resticted to the freshwater mass fo Galindo river, and they do not extend to the rest estuarine waters.

Introducción general

Los estuarios

Los estuarios son zonas de transición entre el mar y el río caracterizadas por un gradiente de las condiciones físico-químicas [1]. Esta característica hace que los estuarios sean sistemas únicos en cuanto a su gran complejidad, variabilidad y dinamismo [1], lo que conlleva a que tengan una de las diversidades de organismos más altas de todo el planeta. En los estuarios habitan desde especies acuáticas estenohalinas y eurihalinas, hasta especies como aves y mamíferos, además de otros seres vivos propios de este ecosistema (como gaviotas, águilas marinas, ratas, castores, etc). Entre ellos son de destacar los microorganismos -bacterias, arqueas, protozoos, etc.- por ser los organismos que se encuentran en la base de la cadena trófica, Son además los organismos que mantienen una relación más directa con el gradiente y las fluctuaciones físico-químicas que ocurren en los estuarios, y por lo tanto, es de esperar que las comunidades de microorganismos se vean afectadas por los diferentes factores que ejercen presión sobre las condiciones ambientales del estuario, como son los tributarios [2], las mareas [3, 4], los vertidos [5, 6], etc.

En cuanto a los tributarios, estos aportan nutrientes desde el interior de los continentes convirtiendo los estuarios en ecosistemas ricos en producción biológica [7] y ejercen un gran efecto sobre la dinámica de los estuarios. De hecho, en periodos de gran descarga de agua de los ríos (agua dulce <0.05 ppt) se da el arrastre de materia terrestre (materia orgánica, minerales, etc) hacia el estuario y un desplazamiento de las masas de agua salinas hacia el mar. Como consecuencia, las comunidades fluviales incrementan su presencia en el estuario, y es de esperar que este tipo de cambios ejerzan cierto efecto sobre los microorganismos de dichos ecosistemas.

Por su parte, el océano y las mareas ejercen gran efecto sobre los estuarios. Por un lado destaca la entrada de las especies planctónicas del océano en los estuarios gracias a las mareas. En cada ciclo mareal las comunidades oceánicas aumentan su presencia en los estuarios mezclándose con las comunidades de agua dulce (<0.05 ppt). Esto ocurre en las masas de agua denominas “de mezcla”, con un rango de salobridad situada entre los valores 0.05-30 ppt, en las que conviven tanto especies de agua dulce como de agua euhalina (>30 ppt). Es por ello que en estas masas de agua “de mezcla” se obtienen los índices de biodiversidad más altos de los ecosistemas estuarinos [8–13]. Por otro lado, destaca el efecto estabilizador que el océano ejerce sobre los estuarios a lo largo del año. La gran masa de agua de los océanos apenas sufre variaciones físico-químicas durante las diferentes estaciones del año, al contrario de lo observado en ríos cuyas variaciones estacionales son acusadas. Por tanto, es de esperar que estas variaciones se vean reflejadas en cambios estructurales y de composición de las comunidades microbianas.

Otro factor importante a considerar en el estudio de los estuarios es, la presión antropogénica. A lo largo de la historia el ser humano se ha asentado en las orillas de estos ecosistemas por diversos intereses; 1) son ecosistemas ricos en alimentos, 2) dan protección natural a las embarcaciones pesqueras, y 3) son enclaves de interés para el comercio marítimo. Así mismo, los asentamientos humanos han alterado estos ecosistemas en mayor o menor grado mediante modificaciones del curso de las aguas con canalizaciones y diques, vertidos industriales y domésticos, y reclamaciones de terrenos inundables como las marismas, urbanización de las orillas, etc [14]. Estas alteraciones, sin duda, han tenido y tienen su efecto directo sobre las diferentes comunidades de los seres vivos que habitan dichos estuarios.

Por estas razones, en un intento por definir los factores clave que determinan las fluctuaciones ecológicas microbianas en los estuarios, y alcanzar una mayor comprensión sobre la interacción entre microorganismos y el medio en el que habitan, nos proponemos caracterizar las comunidades bacterianas estuarinas a lo largo del año. Crearemos así un inventario que

constituya una referencia para sistemas de monitorización. Además, el estudio de las comunidades de dichos hábitats es trascendental, ya que nos da información sobre la calidad de estos ecosistemas y su evolución. Existen estuarios en los que se ha protegido la biodiversidad (reservas de la biosfera, como el de Urdaibai), otros estuarios que están sometidos a programas de recuperación [15] (como el estuario de Bilbao) o, incluso, estuarios que a día de hoy no tienen plan alguno para la saneamiento del impacto antropogénico sufrido. En todos ellos, sea cual sea su estado, el estudio monitorizado de las comunidades microbianas ayudaría a comprender las biodinámicas y tendencias que se estén dando en cada caso [16], y planificar actuaciones en consecuencia con el fin de mejorar la salud de estos hábitats.

Los estuarios de Urdaibai y Bilbao

En este trabajo nos vamos a centrar en los dos estuarios más emblemáticos de la provincia de Bizkaia: El Estuario de Urdaibai y el Estuario de Bilbao. El primero se ubica dentro de una reserva de la biosfera, y está catalogado como estuario de aguas prístinas. El segundo, se ubica en un área altamente urbanizada e industrializada, y está catalogado como estuario con un alto impacto de antropogenización que actualmente está dentro de un plan de recuperación de la calidad de las aguas [17].

Urdaibai es el estuario (43°22'N, 2°43'W) en el que el rio Oka vierte sus aguas al golfo de Bizkaia. Es un estuario pequeño (de un área de 1,89 Km^2 y de 12,5 km de largo), de poca profundidad (3 metros aproximadamente de media) y se caracteriza por ser un estuario meso-macrotidal de aguas bien mezcladas de la zona templada. El ciclo mareal tiene un gran efecto sobre este estuario, ya que con cada bajamar la cuenca se vacía y durante la alta-mar prácticamente toda la masa de agua que cubre el estuario es euhalina (30-35 pp). Esto es debido a la propia dinámica de aguas en este estuario: mientras el flujo mareal es de 240 m^3/seg (cuando está subiendo la

marea), la descarga del rio Oka apenas es de 0,213 m^3/seg [18]. Por lo tanto, con la marea alta el agua salada desplaza el agua dulce hacia el interior. Por otro lado, cabe destacar que el lecho de este estuario es mayoritariamente de arena, pero en su interior, entorno a la zona de marismas, el lecho es fangoso [18], lo cual puede afectar a su comunidad bentónica y acuática [19].

Por otro lado, el estuario de Urdaibai fue calificado como reserva de la biosfera por el comité MaB de la UNESCO en 1984. Urdaibai también está integrado dentro de las convenciones RAMSAR en el 1993, en la Zona de Especial Protección para las Aves (ZEPA) en el 1994 y en la red Natura 2000 en el 2004. Todos ellos constituyen indicadores del entorno ambiental de este estuario. Debido a estas categorías especiales, la caracterización de las comunidades microbianas de dicho estuario es de gran interés, ya que de los resultados y conclusiones obtenidas se puede elaborar planes de calidad de aguas para el seguimiento del carácter ecológico intrínseco de una reserva de la biosfera como es Urdaibai.

Figura 1: Panorámica del estuario de Urdaibai. CC BY-3.0-ES 2012/EJ-GV/Irekia-Gobierno Vasco/Mikel Arrazola.

El Estuario de Bilbao (43º19'N 3º1'W), por otro lado, es un estuario urbano (cruza la zona metropolitana de El Gran Bilbao, que tiene entorno a 1 millón de habitantes) perteneciente también a la costa del golfo de Bizkaia. Es un estuario pequeño (20 km de largo), estrecho (50-2980 metros) y poco profundo (6-30 metros), de estructura macro-mesotidal. Salvo en periodos de grandes lluvias, en las que se producen riadas, el estuario está dominado por aguas euhalinas (30-35 ppt) [20]. El estuario de Bilbao está parcialmente mezclado en la zona exterior y altamente estratificado en el interior. Es decir, en el interior el agua dulce fluye por encima del agua salada y a medida que va avanzando hacia el mar se va diluyendo en el agua salada volviéndose salobre. Esta fuerte estratificación de las aguas interiores se debe a la canalización que sufrió este estuario en el pasado, de manera que la mezcla de agua dulce y salada se prolonga a lo largo de todo el canal [21, 22]. Además de la canalización, la industrialización constituye uno de los impactos antropogénicos más acusados sufridos por el Estuario de Bilbao a lo largo de los últimos siglos; sus orillas y cuencas cercanas fueron testigos de una alta concentración de industria metalúrgica pesada, astilleros y labores de minería [14], convirtiendo al este estuario en uno de los más contaminados de Europa. Esta situación empezó a cambiar en la década de los 80, cuando debido a la reconversión industrial, la mayor parte de la industria pesada de Bizkaia desapareció y comenzó la implantación de planes de recuperación de la ría, la cual sigue en marcha a día de hoy [17]. Es por ello que en la actualidad se observa una mejoría de la calidad del agua en el estuario de Bilbao [23]. Esta mejora de las calidad del agua se ha ido monitorizando desde hace años por parte de diversos investigadores [20-23], sin embargo, nunca se ha llevado a cabo la monitorización de la comunidad bacteriana, siendo este el primer estudio que lo lleva a cabo.

Son varios los tributarios que aportan su agua al estuario de Bilbao: Nervion-Ibaizabal (con un 68% de aporte de agua fluvial al estuario), Kadagua (con un 27%), Galindo (4%), Asua (0.7%) y Gobela (0.3%) [24]. Además, las características físico-químicas de los diferentes aportes de agua dependen de las cuencas de donde provienen. Por ejemplo, el Nervion-Ibaizabal es el tributario más grande, su cuenca abarca 1.900 Km2 en los que además de actividades urbanas hay

actividades agrícolas, ganaderas, canteras de piedra e industrias, tanto químicas como metalúrgicas. El Kadagua por su parte, además de cruzar una zona de actividades ganaderas, también cruza una industria papelera. En las orillas del Asua se agrupan múltiples pequeñas industrias de todo tipo. Por último, El Galindo, además de estar influenciada por una zona minera e industrial, tiene implantado en su orilla una Estación Depuradora de Aguas Residuales (EDAR) que trata el agua doméstica de la zona metropolitana de El Gran Bilbao.

Figura 2: Panorámica del estuario de Bilbao. CC BY-3.0-ES 2012/EJ-GV/Irekia-Gobierno Vasco/.

La importancia de la comunidad bacteriana en los ecosistemas

Las bacterias son seres procariotas unicelulares simples que poseen un número muy limitado de funciones metabólicas. Pese a ello, como las bacterias se organizan en comunidades, el conjunto de estos microorganismos abarca un amplio rango de actividades metabólicas. Este tipo de estructuras ecológicas nos dan a entender que las bacterias también pueden desarrollar interacciones complejas entre ellas. De esta manera, la comunidad bacteriana que hay en cada nicho ecológico adquiere, en gran medida, la capacidad de adaptarse para metabolizar los compuestos que allí se encuentren. Además, estas estructuras complejas proporcionarían mecanismos de resistencia a patógenos, antibióticos y/u otros cambios ambientales. De hecho, las bacterias son capaces de crear biofilms, en los cuales los diferentes tipos de bacterias modifican su metabolismo y se organizan dentro de una matriz extracelular de polisacáridos. Del mismo modo, las bacterias son seres vivos con capacidad de rápida adaptación a los cambios en el ecosistema [25]. Además, ciertas bacterias muestran una alta capacidad de resistencia a los cambios ambientales (resistencia de la membrana celular, resistencia a antibióticos, formas de resistencia, etc). Debido a esta capacidad de adaptación, las bacterias son seres ubicuos en todo el planeta, y se mantienen siempre presentes en el medio [26]. Todas estas características mencionadas hacen de las bacterias un alimento ideal para diferentes seres vivos, como protozoos, fitoplancton, zooplancton y demás filtradores, ubicándolas en la base de las cadenas tróficas de diversos ecosistemas. Este hecho, junto a su capacidad de degradar componentes tanto orgánicos como inorgánicos para volver a introducirlos en las redes tróficas, convierten a las bacterias en seres vivos de gran interés ecológico.

En los estuarios, las comunidades bacterianas están sujetas a una alta variabilidad de condiciones ambientales al pasar de un medio fluvial a un medio oceánico, es decir, de un medio de agua dulce con grandes cambios térmicos a lo largo del año, a al agua salina con cambios térmicos

más ligeros. De hecho, en los ecosistemas estuarinos son de esperar 1) cambios en la comunidad microbiana a lo largo de las diferentes masas de agua, 2) altos niveles de diversidad en el punto donde se mezclan el agua dulce y la euhalina, donde se muestra un gradiente salobre en el que la salinidad se incrementa a medida que se acerca al océano [8–13], 3) encontrar una dinámica estacional específica en cada una de las masas de agua, debido a la gran variación de temperatura que se da en las aguas dulces comparando con las oceánicas [5].

Por otro lado, gracias a la gran capacidad de degradación que tienen las bacterias, el ser humano ha desarrollado diversos sistemas con el fin de aprovechar dicha característica metabólica para llevar a cabo el tratamiento de sus residuos. Por ejemplo, en el tratamiento secundario de las Estaciones Depuradoras de Aguas Residuales (EDAR) se utilizan diversas comunidades bacterianas para convertir los compuestos orgánicos disueltos en el agua en elementos inorgánicos, las que se eliminan por decantación [27, 28]. Gracias a ello el tratamiento de aguas es más efectivo y las aguas vertidas, tras el tratamiento, contienen menos cantidad de contaminantes [27, 28]. Como contrapartida, en el agua vertida de los EDAR, es posible encontrar organismos responsables de su tratamiento secundario [29].

Resumiendo, la importancia ecológica de las comunidades bacterianas dada su situación en la base de las cadenas tróficas, las posibles modificaciones esperadas en su composición taxonómica y funcional en el espacio y tiempo, el posible impacto de su utilización en diferentes procesos antrópicos, etc, nos llevan a acometer un análisis exhaustivo y monitorizado de las comunidades bacterianas en ecosistemas tan dinámicos como son los estuarios, esperando así aumentar nuestra comprensión sobre los factores clave que afectan las fluctuaciones de las comunidades bacterianas, y por ende, aportar datos relevantes sobre los mecanismos de adaptación de las mismas a este ecosistema.

Técnicas de secuenciación masiva orientadas al estudio de las comunidades microbianas

Los desarrollos tecnológicos acaecidos recientemente alrededor de la secuenciación del ADN han abaratado drásticamente sus costes convirtiéndola en una aproximación metodológica económicamente asequible para la mayoría de los grupos de investigación [30]. Del mismo modo, el avance dado en la disciplina de la bioinformática ha permitido el desarrollo de nuevas y específicas herramientas para poder llevar a cabo los diferentes análisis procedentes de las nuevas tecnologías. De esta forma, de entre las aproximaciones metodológicas más utilizadas hoy en día en la ecología microbiana caben destacar la siguientes:

a) Secuenciación de amplicón: Esta aproximación se centra en la amplificación y posterior secuenciación de una región/gen concreta del genoma con el objetivo de obtener la clasificación taxonómica de los seres vivos presentes en una muestra ambiental dada. Para el estudio de la comunidad bacteriana, se utiliza comúnmente la región V4 del gen 16S rDNA [31, 32].

b) Metagenómica: Esta aproximación se basa en la secuenciación de todo el DNA presente en una muestra para su posterior análisis bioinformático que indaga en las funciones/genes potenciales presentes en una comunidad dada [33].

c) Metatranscriptómica: En esta aproximación se secuencia todo el material transcriptómico/los transcritos que haya en una muestra. De esta manera se puede descifrar el metabolismo activo de los organismos presentes en un ecosistema [34].

En un intento por obtener el máximo rendimiento de cada una de estas aproximaciones, el análisis sobre las comunidades bacterianas de los estuarios de Bilbao y Urdaibai se realizará a diferentes niveles:

a) En primera estancia, mediante la técnica del amplicon del 16S (región v4 [35]), se determinarán la estructura/composición taxonómica de los estuarios de Urdaibai y el estuario de Nervion y sus tributarios, estudiando la dinámica bacteriana de cada una de las masas de agua (B30, B33 y B35) a lo largo del ciclo anual.

b) En segunda estancia, se abordarán análisis metagenómicos y metatranscriptómicos de muestras del estuario de Bilbao y su tributarios (Nervion-Ibaizabal y Galindo) en verano, cuando la descarga de los ríos es menos abundante y las masas de agua son más estables. Con ello se pretende (1) detectar la diferenciación metabólica existente a lo largo del estuario, que comienza con agua dulce y finaliza con el agua salada del océano; (2) evaluar la influencia de las descargas del EDAR en las aguas del Galindo.

Objetivos Generales

En la última década los estudios centrados en las comunidades bacterianas, y su ecología, han evolucionado más rápido que en décadas anteriores debido al desarrollo de las técnicas de secuenciación masiva [36–39]. Gracias a ello se han llevado a cabo numerosos estudios en diferentes medios acuáticos como son el océano [39–43], lagos [44–48], ríos [49–52] y ciertos estuarios [8, 12, 53–55]. En este último caso, la mayor parte de los estudios se han llevado a cabo en estuarios grandes con un gradiente salino marcado y bien caracterizado [8, 12, 53–55]. Hasta ahora los estuarios más pequeños han quedado fuera de este tipo de estudios por lo que existe un vacío de conocimiento sobre las comunidades bacterianas de estos ecosistemas. Sin embargo, los estuarios pequeños resultan ser un ecosistema a estudio especialmente interesante dada la alta tasa de cambio esperada en la comunidad bacteriana y su metabolismo como consecuencia de la relativamente drástica transición de agua dulce a agua salina. Por esta razón, el estudio de estuarios de menor entidad, que no muestran un gradiente de salinidad marcado, nos puede aportar información relevante acerca de la capacidad de respuesta de las bacterias a cambios bruscos, en donde la comunidad podría responder modificando su composición taxonómica y/o funcional. Por ello, para poder llegar a entender las dinámicas de cambio referentes a las comunidades bacterianas en los estuarios, los objetivos a alcanzar en este trabajo son los siguientes:

1.- Caracterizar los perfiles taxonómicos de las comunidades bacterianas presentes en cada masa de agua de los estuarios de Bilbao y Urdaibai en cada estación del año. De este modo establecer una referencia de base para posibles análisis posteriores/monitoreos que se realicen de las comunidades bacterianas en dichos estuarios como parámetro adicional a incorporar en las estrategias de monitorización de la calidad del agua y ecosistema que se realizan a día de hoy.

2.- Determinar si en un estuario de drenaje, en el cual su cuenca se vacía en cada bajamar como en el caso de Urdaibai, existe una comunidad bacteriana típica de estuario, o bien si esta comunidad es una prolongación de la comunidad bacteriana típica de aguas oceánicas/costera.

3.- Evaluar el impacto en el estuario de Bilbao de las comunidades bacterianas vertidas por el EDAR situado en las orillas del Galindo, mediante su cuantificación taxonómica y metabólica a lo largo de su cuenca hasta el agua euhalina del estuario.

4.- Desentrañar las vías metabólicas activas durante el periodo de eutrofización que ocurre en verano en el interior del estuario de Bilbao, y de este modo entender el comportamiento de la comunidad bacteriana en dicho periodo caracterizado por niveles de concentración de oxigeno bajo, concentración de clorofila alta y una alta turbiedad del agua.

5.- Analizar los diferentes mecanismos de adaptación de las bacterias al cambio salino, y así, determinar qué conjunto de genes alteran su transcripción al pasar de las aguas dulces a las aguas salinas.

6.- Evaluar la eficacia de cada una de las técnicas utilizadas (amplicón 16S rDNA, metagenómica, metatranscriptómica) y de esta manera poder realizar una comparación posterior de los resultados de cada una de ellas. Ya que al tener las tres técnicas se puede llevar a cabo un análisis más exhaustivo de las comunidades ya que nos permiten medir en cada caso presencia-ausencia, abundancia y actividad metabólica.

General objetives

In the last decade, the studies focused on bacterial communities, and their ecology, have evolved faster than in previous decades due to the development of massive sequencing techniques [36-39]. As a results, numerous studies have been carried out in different aquatic environments such as the ocean [39-43], lakes [44-48], rivers [49-52] and certain estuaries [8, 12, 53-55]. In the latter case, the most of the studies have been carried out in large estuaries with a marked and well characterized saltine gradient [8, 12, 53-55]. So far the smaller estuaries have been left out of this type of studies, so there is a knowledge gap about the bacterial communities of these ecosystems. However, small estuaries turn out to be an especially interesting ecosystem to study, given the high expected rate of change in the bacterial community and its metabolism as a consequence of the relatively drastic transition from fresh water to saline water. For this reason, the study of smaller estuaries, which do not show a marked salinity gradient, can provide us with relevant information about the response capacity of bacteria to sudden changes, where the community could respond by modifying its taxonomic composition and / or functional. Therefore, in order to understand the dynamics of change concerning bacterial communities in estuaries, the objectives to be achieved in this work are the following:

1.- Characterize the taxonomic profiles of the bacterial communities present in each water mass of the estuaries of Bilbao and Urdaibai in each season of the year. In this way, establish a baseline reference for possible subsequent analysis / monitoring carried out of the bacterial communities in these estuaries as an additional parameter to be included in the monitoring strategies for water quality and ecosystem that are carried out to date.

2.- Determine if in a drainage estuary, in which its basin is emptied at each low tide (as in the case of Urdaibai), there is a typical estuarine bacterial community, or if this community is a prolongation of the typical bacterial community of oceanic /coastal waters.

3.- Assess the impact on the estuary of Bilbao of bacterial communities discharged by the WWTP located on the banks of Galindo, through its taxonomic and metabolic quantification along its basin until euhaline-estuarine water.

4.- Unravel the active metabolic pathways during the eutrophication period that occurs in summer in the inner of the Bilbao estuary. In this way, we could understand the behavior of the bacterial community in this period characterized by low oxygen concentration, high chlorophyll concentration and a high turbidity levels on this water mass.

5.- Analyze the different mechanisms of adaptation of the bacteria to salinity change, and thus, determine which set of genes alter their transcription when passing from freshwaters to saline waters.

6.- Evaluate the effectiveness of each of the techniques used (16S rDNA amplicon, metagenomic, metatranscriptomic) and, thus, we can perform a subsequent comparison of the results of each. In addition, having the three techniques available, a more exhaustive analysis of the communities can be carried out, since they allow us to measure in each case presence-absence, abundance and metabolic activity.

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Chapter 1:

Temporal and spatial changes of the prokaryotic community in a drainage estuary: Urdaibai, an UNESCO biosphere reserve

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Abstract

To date, bacterial community studies applying amplicon sequencing approach to drainage estuaries are missing. These estuaries are characterized by relatively short water residence times, and thus, they are considered to be composed of oceanic/coastal bacterial communities. Due to the later, these estuaries are expected not to be significantly affected by terrestrial drivers, anthropogenic pressures or freshwater run-off influences, and therefore, they have received much less research attention than bigger estuaries. In this research study we have focused on the bacterial community of the Urdaibai estuary; a well-flushed drainage estuary that is an UNESCO biosphere reserve. Unexpectedly, despite it empties with each tidal cycle, a differentiated community was found upstream in the inner estuarine water mass. Interestingly, some of the taxa characterizing that water mass community were found to be related either with an anthropogenic (*Enterobacteriaceae, Clostridium, Ruminococcus, Thiothrix*, etc.) or terrestrial origin (*Comamonadaceae, Rhodocyclaceae*, etc.). Expectedly, the downstream outer waters bacterial community was characterized by OTUs (Operational Taxonomic Units) mainly related to the oceanic realm. The evenness and seasonal changes of the community in this outer water, as well as the total bacteria abundance (determined by microscopy), correlated with temperature showing a seasonal regime that might be linked to predation by bacterivorous. Moreover, the bacterioplankton community showed a clear compositional shift between warm and cold periods. All these results suggest that the complexity of the bacterial communities in drainage estuaries is higher than previously considered and that this should be taken into account when designing these system´s water control policies.

Introduction

Coastal areas and estuaries are the greatest biological production zones of the ocean. This is due to physical processes such as up-welling and continental run-off (e.g. [1]). These areas usually show higher species richness than the surrounding waters, and thus, are characterized by complex trophic networks. While in open waters other physicochemical parameters beside salinity (such as temperature, pH, dissolved oxygen concentration, etc.) are very stable, they become highly variable within estuaries (e.g. [2]), generally showing a clear seasonality [3–6]. Nonetheless, the major bacterial plankton phyla and classes are quite stable in different estuarine regions along the word. The occurrence, and even dominance, of *Alphaproteobacteria* has been reported in estuarine regions at variable salinities [7–10]. Also *Gammaproteobacteria* and *Betaproteobacteria* [11–13], together with *Bacteroidetes* [4, 14, 15], are commonly abundant in this habitat.

The study of the bacterioplankton community in such diverse and complex ecosystem is of general interest, since any disturbance, such as eutrophication, can severely alter the system equilibrium potentially leading to a cascade effect [16]. Bacteria represent the base of the aquatic system's trophic chain taking part in different physicochemical processes (metal chelation, carbon and energy flow pathways, etc.) with which they succeed in increasing their biomass [17]. Moreover, bacteria, due to their ubiquity and short life-cycles [18] are the ones to first respond/adapt to any disturbance. Therefore, bacteria could become a useful indicator for the evaluation of water quality in biological monitoring programs [19, 20]. Furthermore, Cloern and colleagues (2016) [21] have recently highlighted that the pace of change in estuarine-coastal ecosystems will likely accelerate as the human population and economies grow and global climate change intensifies. Therefore, the use of bacterial indicators could become essential for anticipating environmental changes with severe ecological, economical and health impacts, and for establishing policies to conserve resources.

Previous studies have generated a growing body of information highlighting the relation of environmental gradients and variations in bacterial production and biomass [22] or community structure at the phylum and sub-phylum level [7, 23] in estuarine environments. However, during the last years high-throughput sequencing technologies have universalized the 16S ribosomal RNA gene (rDNA) amplicon sequencing method, allowing to taxonomically characterize the bacterial community at a greater scale and in more detail. Several studies have already examined the abundance of bacterial taxa (Operational Taxonomic Units or OTUs) in estuaries and coastal areas [8, 14, 24], showing this method to be adequate to analyze these types of ecosystems.

In the present study, the 16S ribosomal RNA gene (rDNA) amplicon sequencing method was used to analyze the dynamics of prokaryotic communities of the Urdaibai estuary along an annual cycle, representing the first study that characterizes the bacterial community of this UNESCO biosphere reserve. Urdaibai is one of the most diverse natural landscapes in the Basque Country. Nevertheless, different anthropogenic impacts occur along this system (e.g. tourist and industrial activities, effluents of Waste Water Treatment Plants (WWTP), agricultural run-off) and they may affect its environmental status [25]. The Urdaibai estuary is a drainage well-flushed system, in other words, the basin empties in each tidal cycle. In this regard, previous works regarding zooplankton [26] and phytoplankton [27] communities showed that the estuary is dominated by coastal species as corresponding to low residence times of the estuarine water mass. With respect to bacteria, species from the sandy riverbed might predominate upstream, although the lack of a differentiated community from that of coastal waters is also likely. Regarding the former, previous studies conducted on the Urdaibai estuary micro and meiobenthos have postulated that the tidal currents cause the resuspension of those organisms within the inner estuarine water column [28, 29]. A similar scenario might be expected for bacteria, where microorganisms from the sandy riverbed might predominate in inner water masses. However, to date, the bacterioplankton communities monitoring studies conducted in this estuary are scarce and mostly limited to the

analysis of total bacterial quantification [30, 31] preventing to test this hypothesis. The present study represents the first high taxonomical resolution level analysis of the Urdaibai estuary´s bacterial community and is intended to fill the gap of knowledge regarding well-flushed estuaries´ bacterioplankton composition and dynamics.

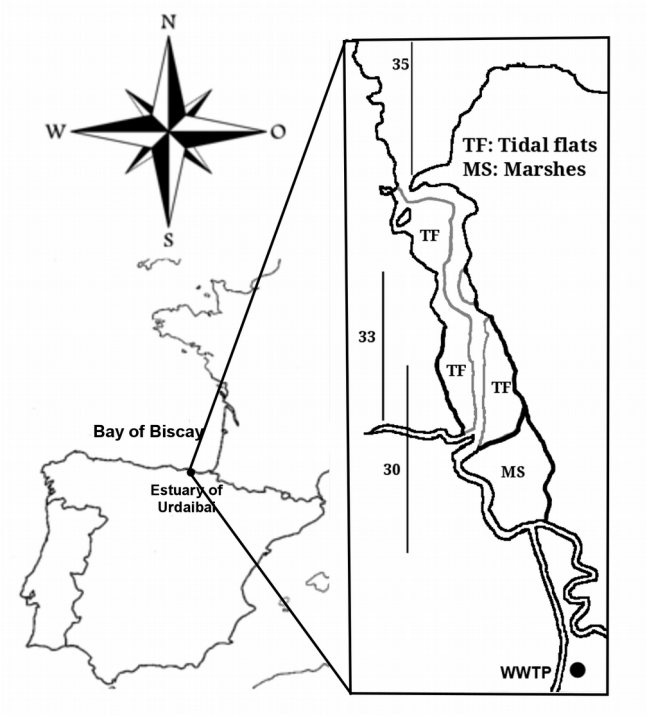
Material and methods

Study area

The estuary of Urdaibai (43°22'N, 2°43'W) drains into the Bay of Biscay, being the last tract of the Oka river. This estuary is a temperate, small (area ≈ 1.89 km^2, ~12.5 km long), shallow (average depth of 3 m), meso-macrotidal system. Its bed is mostly sandy, but inside, near the marshes (Fig. 1), the bottom is muddy [32]. The estuary is a well-flushed system except for occasional stagnant waters [33, 34], emptying in each tide cycle and thus the water column is strongly mixed [35]. At high tides, estuarine water masses are mostly euhaline (30-35 ppt) (Fig. 1). This is due to the system´s geomorphology, the low river discharge (average discharge = 0.213 m^3/s) and the relatively high tidal flow at neap tides (max. 240 m^3/s) [36]. In 1984, the estuary of Urdaibai was granted an UNESCO Biosphere Reserve status; in 1993, it was included in the RAMSAR list, and in 2004 in the Natura 2000 European Network (ES 0000144 SPA and ES 2130007 SAC, respectively).

Sample collection was carried out monthly from September 2013 to September 2014. In total, 72 samples (including two replicates at each sampling point) were collected for the 13-months period. Sampling took place only on days of neap tide coefficient (30-50), always at high tide, and at approximately the same time of the day (10:00 AM-12:00 PM CET) to avoid confounding variables. Water masses of salinity 30, 33 and 35 ppt were localized along the Urdaibai estuary (herein after U30, U33 and U35) and samples were collected at a middle depth (~1 m), where the water had the required salinity in the whole column (Fig. 1).

Fig. 1. Estuary of Urdaibai. The localization of the three sampled water masses (salinity 30, 33 and 35 ppt) at high tide conditions is delimited with vertical lines. In addition, the WWTP (Waste Water Treatment Plant) location is indicate in the map.



Samples were collected using an oceanographic Niskin bottle. The water (10 L approx.) was stored in opaque plastic jerry cans in the field. Once in the laboratory, the water was filtered (5 L approx.) through 20 µm Nylon net filters (Millipore, 90 mm diameter) and bacteria were collected with 0.22 µm Durapore® membrane filters (Millipore, 47 mm diameter). Filtration was performed in triplicate using a Kitasato Flask and a vacuum pump. The whole process, from sampling to storage, took less than 3 hours to perform. All filters were store at -80 ºC until DNA extraction.

At each sampling point vertical profiles (every 0.5 m) of salinity, temperature, pH, and dissolved oxygen saturation (DO %) were obtained in situ using a YSI 556 MPS Multiparameter Probe. Water transparency was measured with a Secchi Disk. Chlorophyll concentrations were calculated from spectrophotometric measurements on acetone extracts using a monochromatic method with acidification [37]. In addition, river discharge (Qms) data was obtained through the

Hydrometeorology Service of the Regional Council of Bizkaia (http://www.bizkaia.eus/Ingurugiroa_Lurraldea/Hidrologia_Ac/Datos_meteo.asp?Idioma=CA&Tem_Codigo=2679), concretely from the Muxika station.

DNA extraction

Total genomic DNA was extracted from one half of the 0.22 μm Durapore® membrane filters using PowerSoil DNA isolation kit (MoBio laboratories, Inc., Carlsbad, CA, USA) following the manufacturer protocol. The DNA quantity and quality of each sample was assessed by either a ND-1000 spectrophotometer (NanoDrop) or Qubit fluorimeter (Life technologies). To avoid cross-contamination all tools were flame-sterilized between samples and laboratory surfaces were decontaminated with DNA-ExitusPlus (Applychem) after each session. Finally, the DNA extractions were stored at -20 ºC until DNA sequencing.

16S rDNA amplification and sequencing

The 16S rDNA gene of the 72 samples was amplified and sequenced by the Next Generation Sequencing Core at Argonne National Laboratory, Lemont, IL (USA) (http://www.earthmicrobiome.org/). Earth Microbiome Project's protocols were applied for the amplification and sequencing of the community 16S rDNA gene v4 region by using 515f and 806r primers that contained 12 bp Golay-barcodes for sequencing [38]. The sequencing was carried out in two MiSeq runs (2x150 paired-end). The data is available in QIITA portal (ID 10470) and ENA database (study: PRJEB14094).

Bioinformatic pipeline

Raw sequences were trimmed using Sickle tool (v1.33) [39] with default parameters (including a Phred score ≥ 20). Paired-end reads were first merged with Pear software (v0.9.6) [40] using a cut-off of 0.01 (P-value) for the observed expected alignment score. Then, to remove non-existent barcodes from the fastq file achieved by Pear, fastq-barcode.pl [41] was used. Chimera sequences were removed by identify_chimeric_seqs.py in QIIME (v1.9: [42]) using the usearch algorithm' (v7.0.1090) *de novo* method [43, 44]. Sequences that were 240-260 bp in length (average 253 bp) were taken into account in the subsequent analyses, to avoid background noise. An open reference OTU picking method was used in QIIME software (v1.9: [42]) for clustering, using a 97% similarity cut-off in the UCLUST algorithm (v1.2.22q) [43] and the taxonomy of the reference sequences was assigned based on Silva 119 database version (Quast et al. 2013).. In this process, sequences that failed in PYNAST alignment were omitted from the OTU table. Then, all chloroplast were removed from the BIOM file using filter_taxa_from_otu_table.py script in QIIME. Afterwards, samples with less than 5000 sequences were eliminated and consecutively, every OTUs with less than 10 sequences were removed. Finally, the BIOM file was normalized using metagenomeSeq's CSS algorithm, which normalized sequences using the cumulative sum scaling transformation [45].

Data analysis

Correlations within physical-chemical variables were check using FactoMineR (v1.31.5) package [46]. The resulting Principal Components Analysis (PCA) enables removing the variables that have a low statistical weight, or show to be correlated with other variable. In addition, the PCA contributes to the depiction of the physico-chemical dynamics and it eases finding putative drivers of bacterial community's changes between water masses.

To identify which environmental variables from the ones tested in the study (salinity, temperature, pH, DO%, water turbidity, precipitation and Chlorophyll) are related to community changes, Spearman's rank correlation coefficient (rho) was carried out. The impact of these environmental factors on the bacterial community was analyzed using the bio-env method of vegan (v. 2.3-4) R package [47]. This method makes Spearman's rank correlations between the community distance matrix and the euclidean environmental distance matrix, ranking the environmental variables by their importance. This analysis was carried out in cumulative format, thus taking into account the cumulative effect of the environmental variables on microbial communities. In order to calculate the percentage of beta diversity variation explained by temperature, an analysis of Adonis was performed. In addition, to identify the OTUs whose abundances significantly changed according to temperature, Spearman analysis was carried out.

To determine the community dissimilarity along the annual cycle in all water masses, a Bray-Curtis distance network was carried out using phyloseq (v. 1.14) R package [48]. Beta diversity estimates based on Bray-Curtis distances were used to examine community dissimilarity and community changes across seasons. Alpha diversity (Shannon) of these samples was calculated using phyloseq (v1.14) R package [48].

To describe the community changes in the different water masses of this estuary, the taxonomic groups that reached 1% of the total of abundance in the community had been taken into account. The abundance variation of these taxa was analyzed along the time, to determine putative blooms of particular groups.

To visualize the bacterial community composition of the samples, tables summarizing the taxa found were generated in QIIME v1.9 software [42]. Next, to identify the characteristic community of each water mass, a supervised learning analysis was performed for each water mass, using the

Random Forests classifier [49, 50], using ten-fold cross-validation model with 1,000 trees, with OTUs as predictors and the water masses (U30, U33, U35) as class label. This method determines the diagnostic power of bacterial profiles for predicting the entity of the water masses by using a subset of samples to train a model that identifies unique features within data categories. The technique then determines the accuracy of the model by categorizing sample subsets that were not used to build the model. This way, the discriminative power of the microbial community of each group (water mass) and the robustness of the groupings themselves were evaluated. Analysis of Similarity (ANOSIM) statistics (999 permutations) were carried out with the ANOSIM function [47] and were used to test whether the bacterial community differs significantly according to the different water masses. In order to determine water mass specific OTUs, as well as the OTUs that were present in all three salinity ranges, the within water masses core microbiome was analyzed. The core microbial community was defined as the OTUs that were present in at least 95% of the samples in each water mass (U30, U33 and U35) along the year. To compare the shared OTUs between the different water masses, a Venn diagram tool was used (http://bioinformatics.psb.ugent.be/webtools/Venn/). Finally, to identify the characteristic OTUs of the inner and the outer water masses Kruskal-Wallis statistical method was carried out among the water masses communities.

Microscopy-based abundance

Bacterial abundance at U35 samples (coastal water with 35 ppt) was quantified using epifluorescence microscopy (30-33 ppt salinities water samples were not checked to avoid the distortion caused by the resuspension of the sand of the bed of the estuary in those samples). To quantify bacterial abundances, filters (the remaining half not used at the DNA extraction step) were individually placed in 5 ml of sterile saline solution (1.9% w/v NaCl) and vigorously vortexed for 3 min. Then, samples were sonicated in an ice bath for 20 s with a Sonic Vibra Cell (Sonic &

Materials, Inc.) at 100 W and newly vortexed. Finally, the supernatant that contained the detached bacteria was collected and total bacteria were enumerated according to the procedure described by Hobbie and colleagues [51]. More in detail, aliquots of the processed samples were filtered throughout 0.22 μm-pore-size black polycarbonate filters (Millipore, 25 mm diameter) and stained with acridine orange (0.01%, w/v, final concentration) for 2 minutes. The stained filters were examined under a Nikon epifluorescence microscope equipped with a filter block B-2A (EX450-490 excitation filter, DM505 dichroic mirror and BA520 barrier filter). Total numbers of bacteria were estimated by counting a minimum of 20 fields.

Results

After removing OTUs that assigned to chloroplast (9.83% of the reads), those with less than 10 counts and after removing samples with less than 5,000 total reads (4 samples in total) the total number of 16S rDNA gene sequences in the remaining 68 samples was 2,029,091, from which a total of 6355 OTUs had been identified.

The effect of the environmental variables in the microbial communities

The correlation analysis among the environmental variables showed a similar pattern for U30 and U33 water masses, but that differed in U35 (Supplementary 1). Interestingly, a correlation between temperature and chlorophyll was reported in the PCA in inner water masses (U30 and U33), where both variables were in negative relationship to the river discharge (Qms). Moreover, DO % and pH grouped together and formed a distinct group in those water masses (Supplementary 1). However, in outer waters (U35) temperature and chlorophyll were not correlated, and temperature variations appeared more related to DO%.

Spearman´s rank correlation results evidenced lower correlation values among environmental variables and microbial composition in the outer water mass (U35) (Spearman´s rho< 0.55 in U35 and Spearman´s rho> 0.72 in U30 and U33). Temperature and river discharge showed highest and significant Spearman rho values for all water masses (U30, U33, U35) (Supplementary 2). Additionally, Adonis test showed that approximately 19% of the variation of the microbial communities was explained by temperature (R^2= 0.19, p< 0.001). Interestingly, Spearman analysis evidenced that a large number of OTUs (n= 142) correlated with temperature changes occurring throughout the year (rho> 0.7) in the whole estuary (Supplementary 3). For instance, members of *Actinobacteria* and *Flavobacteria* were more common in high temperature months (spring-summer-

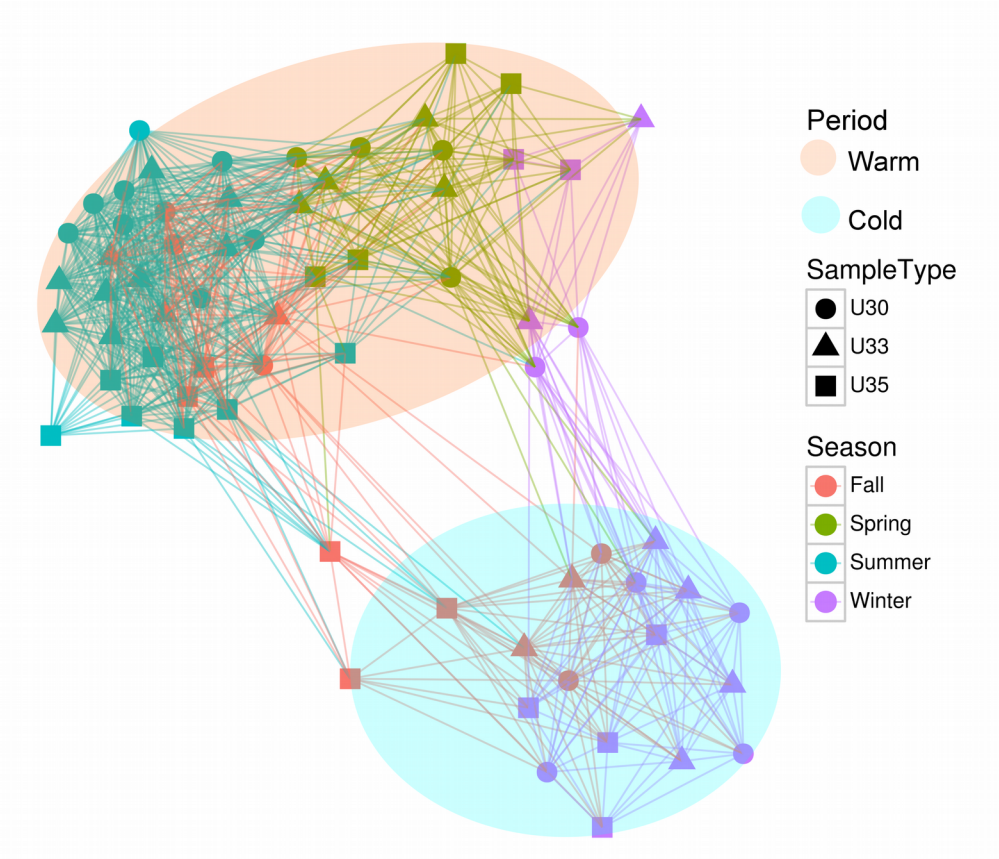
early fall), herein warm period. To the contrary, some of the OTUs affiliated with *Campylobacterales*, *Aeromonadales*, *Pseudomonadales* and *Vibrionales* orders were more abundant during the cold months (late fall-winter), herein cold period.

Seasonal effects into microbial communities

Bray Curtis distance network evidenced seasonality where two main periods, hereafter “warm” and “cold”, were identified for bacterial communities independently of the salinity (figure 2): spring, summer and early fall months (warm period) and late fall and winter months (cold period).

The abundances of many prokaryote taxa changed between cold and warm seasons, as shown in Table 1. Archaea relative abundances fluctuated considerably along the year, with higher densities detected during the cold period. Gamma- and *Alphaproteobacteria* represented together more than half of the total bacterial relative abundance and showed a temporal distribution; *Gammaproteobacteria* dominated along the cold period and *Alphaproteobacteria* (principally *Rhodobacteraceae* and *Pelagibacteraceae*) during spring and summer (warm period). Other taxa also showed a clear seasonality: *SAR406*, *Bacteroidales*, *Campylobacterales*, *Acidimicrobiales* and *Pseudomonadales* were more abundant in the cold period, while *Microbacteriaceae* and *Flavobacteriales* percentages increased in the warm period (Table 1, Supplementary 4).

Fig. 2. Annual dynamic of bacterial communities of the estuary of Urdaibai. Bray-Curtis distance network analysis depicted two main clusters, where samples' bacterial community could be classified into a Warm and Cold period. Samples are coloured according to season and water masses are represented by symbols (round, triangle and square for U30, U33 and U35, respectively). Only connecting edges with lower values than 0.6 Bray-Curtis distance are represented.



					U30			U33			U35		
Kingdom	Phylum	Class	Order	Family	Anual	Cold	Warm	Anual	Cold	Warm	Anual	Cold	Warm
k__ARCHAEA					0.86 %	1.46 %	0.49 %	1.06 %	1.46 %	0.82 %	1.77 %	3.01 %	0.99 %
	p__Euryarchaeota				0.65 %	0.95 %	0.47 %	0.83 %	0.97 %	0.75 %	1.36 %	2.06 %	0.92 %
		c__Thermoplasmata			0.65 %	0.95 %	0.47 %	0.83 %	0.97 %	0.75 %	1.36 %	2.06 %	0.92 %
			o__E2		0.65 %	0.95 %	0.47 %	0.83 %	0.97 %	0.75 %	1.36 %	2.06 %	0.92 %
				f__Marine group II	0.62 %	0.88 %	0.47 %	0.80 %	0.89 %	0.75 %	1.30 %	1.92 %	0.91 %
k__BACTERIA					96.87 %	97.36 %	96.57 %	96.79 %	97.19 %	96.53 %	96.47 %	95.66 %	96.98 %
	p__Proteobacteria				66.30 %	67.86 %	65.32 %	66.07 %	67.87 %	64.95 %	66.91 %	66.08 %	67.44 %
		c__Alphaproteobacteria			28.32 %	24.13 %	30.95 %	29.59 %	23.57 %	33.35 %	31.09 %	25.09 %	34.83 %
			o__Rhodobacterales		19.70 %	16.49 %	21.71 %	20.20 %	16.22 %	22.68 %	17.79 %	13.53 %	20.46 %
				f__Rhodobacteraceae	19.42 %	16.20 %	21.44 %	19.89 %	15.90 %	22.38 %	17.29 %	12.94 %	20.01 %
			o__Rickettsiales		2.47 %	2.44 %	2.49 %	2.81 %	2.31 %	3.13 %	4.52 %	4.00 %	4.85 %
				f__Pelagibacteraceae	1.76 %	1.49 %	1.92 %	2.04 %	1.38 %	2.45 %	3.40 %	2.61 %	3.90 %
		c__Gammaproteobacteria			23.37 %	26.62 %	21.34 %	23.38 %	26.24 %	21.59 %	27.09 %	29.31 %	25.71 %
			o__Alteromonadales		10.79 %	10.16 %	11.18 %	11.62 %	11.90 %	11.44 %	10.63 %	10.05 %	11.00 %
				f__Alteromonadaceae	4.02 %	4.34 %	3.81 %	4.22 %	4.47 %	4.06 %	4.39 %	4.02 %	4.63 %
				f__HTCC2188	2.98 %	2.64 %	3.19 %	2.64 %	2.43 %	2.78 %	1.80 %	1.86 %	1.77 %
				f__OM60	2.86 %	1.76 %	3.55 %	2.97 %	1.81 %	3.70 %	2.88 %	2.29 %	3.24 %
			o__Oceanospirillales		5.25 %	6.41 %	4.53 %	5.49 %	5.92 %	5.22 %	8.72 %	9.07 %	8.50 %
				f__Oceanospirillaceae	2.00 %	2.75 %	1.53 %	2.09 %	2.52 %	1.83 %	3.61 %	3.42 %	3.74 %
				f__Halomonadaceae	1.62 %	2.22 %	1.25 %	1.76 %	1.88 %	1.69 %	3.33 %	3.80 %	3.04 %
			o__Vibrionales		0.75 %	0.81 %	0.71 %	0.72 %	0.85 %	0.64 %	1.89 %	1.33 %	2.23 %
			o__Chromatiales		1.34 %	1.20 %	1.42 %	1.20 %	1.20 %	1.20 %	0.80 %	1.29 %	0.49 %
			o__Thiotrichales		0.87 %	1.25 %	0.63 %	0.88 %	1.16 %	0.71 %	1.32 %	2.16 %	0.79 %
				f__Piscirickettsiaceae	0.70 %	1.00 %	0.52 %	0.74 %	0.95 %	0.60 %	1.13 %	1.80 %	0.71 %
			o__Pseudomonadales		1.24 %	2.36 %	0.54 %	1.08 %	2.09 %	0.44 %	0.72 %	1.06 %	0.51 %
		c__Betaproteobacteria			8.96 %	9.25 %	8.78 %	7.86 %	9.73 %	6.69 %	4.05 %	4.57 %	3.73 %
			o__Burkholderiales		5.22 %	5.36 %	5.14 %	4.45 %	5.76 %	3.62 %	2.09 %	2.24 %	2.00 %
				f__Comamonadaceae	4.86 %	4.92 %	4.82 %	4.12 %	5.28 %	3.39 %	1.89 %	2.03 %	1.81 %
			o__Methylophilales		1.55 %	1.27 %	1.73 %	1.46 %	1.21 %	1.62 %	0.96 %	0.88 %	1.00 %
				f__Methylophilaceae	1.54 %	1.26 %	1.72 %	1.45 %	1.18 %	1.62 %	0.96 %	0.88 %	1.00 %
			o__Rhodocyclales		1.31 %	1.72 %	1.06 %	1.15 %	1.80 %	0.74 %	0.45 %	0.73 %	0.28 %
				f__Rhodocyclaceae	1.31 %	1.72 %	1.06 %	1.15 %	1.80 %	0.74 %	0.45 %	0.73 %	0.28 %
		c__Epsilonproteobacteria			3.13 %	4.89 %	2.02 %	2.90 %	5.20 %	1.47 %	2.57 %	3.28 %	2.13 %
			o__Campylobacterales		3.13 %	4.89 %	2.02 %	2.90 %	5.20 %	1.47 %	2.57 %	3.28 %	2.13 %
				f__Campylobacteracea	2.57 %	4.05 %	1.65 %	2.37 %	4.34 %	1.14 %	2.27 %	2.71 %	2.00 %
		c__Deltaproteobacteria			2.49 %	2.97 %	2.20 %	2.32 %	3.12 %	1.83 %	2.09 %	3.83 %	1.01 %
	p__Bacteroidetes				21.36 %	19.75 %	22.37 %	21.67 %	19.29 %	23.16 %	20.01 %	17.76 %	21.41 %
		c__Flavobacteriia			17.20 %	14.01 %	19.19 %	17.80 %	13.56 %	20.45 %	17.04 %	13.89 %	19.01 %
			o__Flavobacteriales		17.20 %	14.01 %	19.19 %	17.80 %	13.56 %	20.45 %	17.04 %	13.87 %	19.01 %
				f__Flavobacteriaceae	8.80 %	7.49 %	9.62 %	9.33 %	7.22 %	10.65 %	9.09 %	7.17 %	10.29 %
				f__Cryomorphaceae	6.75 %	5.08 %	7.79 %	6.82 %	5.06 %	7.92 %	5.87 %	4.76 %	6.56 %
		c__Bacteroidia			2.27 %	4.11 %	1.11 %	2.02 %	4.05 %	0.74 %	1.05 %	1.75 %	0.62 %
			o__Bacteroidales		2.27 %	4.11 %	1.11 %	2.02 %	4.05 %	0.74 %	1.05 %	1.75 %	0.62 %
	p__Actinobacteria				4.90 %	2.93 %	6.13 %	4.69 %	3.16 %	5.65 %	3.39 %	3.38 %	3.40 %
		c__Actinobacteria			3.87 %	1.33 %	5.47 %	3.57 %	1.65 %	4.77 %	1.36 %	0.59 %	1.84 %
			o__Actinomycetales		3.83 %	1.23 %	5.45 %	3.53 %	1.56 %	4.76 %	1.32 %	0.55 %	1.81 %
				f__Microbacteriaceae	3.61 %	1.12 %	5.16 %	3.31 %	1.41 %	4.49 %	1.11 %	0.35 %	1.59 %
		c__Acidimicrobiia			1.01 %	1.58 %	0.66 %	1.09 %	1.44 %	0.87 %	1.96 %	2.71 %	1.50 %
			o__Acidimicrobiales		1.01 %	1.58 %	0.66 %	1.09 %	1.44 %	0.87 %	1.96 %	2.71 %	1.50 %
				f__OCS155	0.72 %	1.08 %	0.50 %	0.83 %	0.98 %	0.73 %	1.42 %	1.65 %	1.28 %
	p__Verrucomicrobia				0.92 %	0.76 %	1.02 %	0.81 %	0.69 %	0.88 %	1.17 %	1.22 %	1.13 %
	p__Cyanobacteria				0.42 %	0.57 %	0.34 %	0.63 %	0.56 %	0.68 %	1.69 %	1.17 %	2.02 %
		c_Synechococcophycideae			0.38 %	0.51 %	0.30 %	0.59 %	0.48 %	0.66 %	1.66 %	1.13 %	1.99 %
			o__Synechococcales		0.38 %	0.51 %	0.30 %	0.59 %	0.48 %	0.66 %	1.66 %	1.13 %	1.99 %
				f__Synechococcaceae	0.38 %	0.51 %	0.30 %	0.59 %	0.48 %	0.66 %	1.66 %	1.13 %	1.99 %
	p__SAR406				0.44 %	0.92 %	0.13 %	0.48 %	0.83 %	0.26 %	1.05 %	1.88 %	0.53 %
		c_AB16			0.44 %	0.92 %	0.13 %	0.48 %	0.83 %	0.26 %	1.05 %	1.88 %	0.53 %

Table 1. Relative abundance variations of bacterial taxonomic groups between cold and warm periods. The mean relative abundance of each taxonomy level in the annual cycle for cold (late fall and winter months) and warm periods (spring, summer and early fall months), within each water mass (U30, U33, U35). Only the bacterial groups that at some point reached 1% of the total community abundance are shown.

In addition, the total bacteria abundance (count per liter) counted by fluorescence microscopy in U35 samples, decreased along fall and remained low until February, when it increased and remained stable until September (Supplementary 5). In the same way, the bacterial abundances of U35 increased in April, while the alpha diversity and evenness calculated from amplicon sequencing data for that month decreased. In this regard, the bacterial counts and the alpha diversity (Shannon) values had a negative correlation (Spearman´s rho= -0.6). When the overall bacterial abundance decreased, the number of identified OTUs significantly increased and, such, the evenness of the community of the sample.

Spatial distribution of the bacterial community

Proteobacteria, mainly Alpha- (29.66%) and *Gammaproteobacteria* (24.61%), was by far the dominant phylum, including more than 66% of the total OTUs, followed by *Bacteroidetes* (21.01%) and *Actinobacteria* (4.07%). The bulk of OTUs were classified into the following orders: *Rhodobacterales* (19.23%), *Flavobacteriales* (17.35%), *Alteromonadales* (11.01%), *Oceanospirillales* (6.49%) and *Burkholderiales* (3.92%). In these taxonomic groups some families stood out due to their higher abundances: *Rhodobacteraceae* (*Rhodobacterales*) was the most abundant family (18.87% of the total microbial community) followed by the *Flavobacteriia Flavobacteriaceae* (9.07%) and *Cryomorphaceae* (6.48%). Moreover, *Alteromonadaceae* (4.21%),

OM60 (2.9%) and *HTCC2188* (2.47%) clades and *Comamonadaceae* (3.63%) stood out within *Alteromonadales* and *Burkholderiales*, respectively (Supplementary 4).

When extending the analysis to minor abundant taxa, those OTUs included both prokaryotes implied in general processes, as biogeochemical cycles, and those related to specific characteristics of the estuary, as WWTP related bacteria. Examples of the first case were, among others, the sulfur-reducing bacteria (*Desulfobacterales*, *Desulfovibrionales* or *Desulfuromonadales*) and the prokaryotes implied in the cycling of nitrogen (*Nitrospirales*, *Nitrosomonadales* or *Thaumarchaeota*), which were detected in all water masses. OTUs assigned to *Thiothrix*, *Zoogloea*, *Enterobacteriaceae*, *Clostridium* or *Ruminococcus*, typical in waste-water treatment of WWTP, were also identified (Supplementary 6).

Regarding community changes along the salinity gradient, each water mass had its particular community composition (Table 1). For example, the *Cyanobacteria* counts, mainly represented by OTUs affiliated with *Synechococcus* genus, increased with the salinity, representing the 0.42%, 0.63% and 1.69% of the total OTU number in U30, U33 and U35 samples, respectively. Differences could be also observed in the *Actinobacteria* phylum, which accounted for 3.39% (U35) to 4.9% (U30) of the total bacterial abundance. The most abundant families within this phylum, *Microbacteriaceae* and *OCS155*, showed an opposite behaviour. While the highest abundance of *OCS155*-related OTUs occurred in U35 (1.42%), *Microbacteriaceae*-related OTUs decreased as salinity increased. In this regard, *Microbacteriaceae* was the sixth more abundant family in U30 (3.61%) and U33 (3.31%) but the eleventh in U35 (1.11%). Similarly, *Comamonadaceae* family experimented a clear decrease in its abundance from U30 (4.86%) to U35 (1.89%), which greatly explained the gradual decrease that was undergone by *Betaproteobacteria* from inner (8.96%) to outer waters (4.05%).

To analyse the community dissimilarity level, the Random Forests supervised learning model revealed that the observed microbiota had a high discriminative power to distinguish samples as coming from U35 (< 0.05 classification error), while U30 and U33 had the highest predictive errors (> 0.6), as shown in Fig. 3A, as there is a cross classification between U30 and U33 samples. For instance, fifteen U30 samples got incorrectly classified as U33, and the same occurred to fourteen U33 samples, that were misclassified as U30 (Fig. 3A). Similarly, ANOSIM test showed that while there was a significant community composition difference between the inner waters (U30 and U33) and U35 ($R= 0.26$, $p< 0.001$), no significant differences were found between U30 and U33 masses bacterial communities ($R= -0.01$, $p= 0.61$).

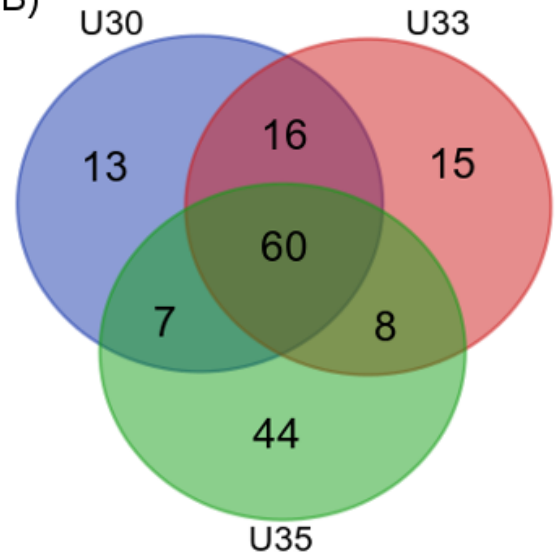
Each water mass showed high percentage of OTUs present in 95% of its samples (Supplementary 7); 66.03% of total number of OTUs in U30, 67.68% in U33 and 63.19% in U35, reflecting the high bacterial community stability in these water masses. Moreover, the inner and outer waters shared 60 core OTUs that represented the 52.36 ± 4.02% of the total abundance of the microbial community of the estuary. The inner water masses (U30 and U33), shared an additional 2.54 ± 0.29% of the total community abundance in which OTUs with the following taxonomic classification were identified: *Comamonadaceae* (0.19% of bacterial abundance, 3 OTUs), *HTCC2188* (0.28%, 3 OTUs), *Microbacteriaceae* (0.94%, 2 OTUs) and *Rhodobacteraceae* (0.57%, 4 OTUs), 1 OTU affiliated to *Helicobacteraceae* (0.06%) and another OTU to *Betaproteobacteria* (0.12%). These water masses had their own characteristic OTUs with 2.25% (13 OTUs) of the abundance of the community of U30 being unique for this water mass and 1.28% (15 OTUs) just present in U33 (Fig. 3B). Meanwhile, U35 had the highest amount of unique bacteria, 6.05% (44 OTUs) of abundance in its community. Among these OTUs, the following families predominated: *Flavobacteriaceae* (1.44% of the bacterial abundance, 6 OTUs), *Oceanospirillaceae* (1.58%, 5 OTUs) and *Rhodobacteraceae* (0.45%, 7 OTUs).

Fig. 3. The confusion matrix for water masses and the Venn diagram for the core OTU for each water mass (all samples of the annual cycle). A) A Random forests classification analysis was conducted based on the communities' dissimilarities among water masses. In this confusion matrix, the first column refers to where the samples were collected, while row numbers indicate the number of samples that are predicted to belong to each water mass. The classification error value is the rate of misclassified samples within each mass. B) Venn diagram for each water mass core-OTU (OTUs appearing in the 95% of samples), throughout the year. The diagram shows shared core microbiota among water masses, as well as the percentage of unique OTUs in each water mass.

A)

True\Predicted	U30	U33	U35	Class error
U30	7	15	1	0.6957
U33	14	9	0	0.6087
U35	0	1	21	0.0455

B)



When analyzing the characteristic OTUs of the three different water masses, Kruskal-Wallis statistical test detected OTUs assigned to *Oceanospirillaceae* (Otu_8, 11, 16-18, 23, 28), *Flavobacteriaceae* (Otu_9) *Pelagibacteraceae* (Otu_6, 30) or *Synechococcus* (Otu_4, 27, 32) to be significantly more abundant in U35 water mass, while higher abundances of OTUs within

HTCC2188 clade (Otu_20, 24), *Alteromonadaceae* (Otu_34) and *Comamonadaceae* (Otu_7, 25-26, 33, 36) families relate more to the inner water mass (Table 2).

OTU	U30_mean	U33_mean	U35_mean	taxonomy
Otu_1	6,83	7,276	8,511	f__Rhodobacteraceae
Otu_2	5,073	5,623	7,739	c__Alphaproteobacteria
Otu_3	5,168	5,612	7,721	o__Acidimicrobiales; f__OCS155
Otu_4	3,656	4,766	7,537	f__Synechococcaceae; g__Synechococcus
Otu_5	3,203	3,128	6,928	f__Pseudoalteromonadaceae; g__Pseudoalteromonas
Otu_6	4,097	4,374	6,289	f__Pelagibacteraceae
Otu_7	9,282	8,925	6,218	f__Comamonadaceae; g__RS62
Otu_8	1,854	2,266	6,062	f__Oceanospirillaceae; g__Oleispira
Otu_9	3,968	4,364	5,792	f__Flavobacteriaceae
Otu_10	2,71	3,6	5,648	f__HTCC2089
Otu_11	1,644	1,914	5,011	f__Oceanospirillaceae
Otu_12	2,07	2,825	4,815	f__Cryomorphaceae; g__Fluviicola
Otu_13	2,084	1,686	4,513	f__Vibrionaceae
Otu_14	1,795	2,328	4,049	f__OM60
Otu_15	1,292	1,757	3,592	c__Alphaproteobacteria
Otu_16	0,721	1,01	3,477	f__Oceanospirillaceae
Otu_17	0,943	0,941	3,446	f__Oceanospirillaceae; g__Oleispira
Otu_18	0,857	1,328	3,363	f__Oceanospirillaceae
Otu_19	0,587	0,982	2,316	c__Alphaproteobacteria
Otu_20	4,908	4,435	2,278	f__HTCC2188; g__HTCC
Otu_21	4,839	4,042	1,76	c__Betaproteobacteria
Otu_22	0,344	0,037	1,74	f__Pseudoalteromonadaceae; g__Pseudoalteromonas
Otu_23	0,248	0,28	1,738	f__Oceanospirillaceae
Otu_24	3,959	3,463	1,614	f__HTCC2188; g__HTCC
Otu_25	4,261	3,741	1,503	f__Comamonadaceae
Otu_26	4,112	3,429	1,146	f__Comamonadaceae
Otu_27	0,083	0,195	1,126	f__Synechococcaceae; g__Synechococcus
Otu_28	0,21	0,045	0,95	f__Oceanospirillaceae; g__Oleispira
Otu_29	0,201	0,035	0,898	f__Halomonadaceae; g__Candidatus Portiera
Otu_30	0	0	0,894	f__Pelagibacteraceae
Otu_31	0	0	0,805	f__Phyllobacteriaceae
Otu_32	0	0	0,578	f__Synechococcaceae; g__Synechococcus
Otu_33	2,781	2,376	0,499	f__Comamonadaceae
Otu_34	2,872	2,261	0,222	f__Alteromonadaceae; g__BD2-13
Otu_35	1,771	1,13	0,05	f__Rhodobacteraceae; g__Rhodobacter
Otu_36	1,324	0,82	0,04	f__Comamonadaceae

Table 2: Characteristic OTUs between the different water masses. Mean relative abundance (%) values, per OTU, obtained by Kruskal-Wallis analysis are shown. Only OTUs with Bonferroni values lower than 0.05 are represented. Shadowed cells indicate the greater abundance of each OTU between the water masses.

Discussion

Urdaibai is a well-flushed draining estuary that empties with each tide cycle [35]. Thus, the three water masses (U30, U33, U35) analysed in this study had a highly similar microbiome community, as shown in figure 3. This is due to the particular water dynamic of this estuary, where euhaline waters (salinity ppt> 30) dominate within it. The abundance of marine-associated taxa as *Rhodobacterales* and *Gammaproteobacteria* [4] in the samples suggests that this euhaline habitat is highly influenced by oceanic populations. Nevertheless, typical freshwater bacteria as those affiliated within the *Betaproteobacteria* [4, 52, 53] co-occurred with marine prokaryotes, especially in the inner water masses (Table 1, Supplementary 4), indicating that freshwater and marine bacterioplankton communities mix along this well-mixed system; a pattern previously reported in stratified estuaries [23, 54]. Thus, the typical sea inlet characteristic was observed in Urdaibai, despite being a drainage estuary.

The community composition of Urdaibai resembled to that observed in locations relatively rich in organic carbon [14, 55–57]. In all samples, regardless of the month or water mass, *Rhodobacteraceae* was by far the most abundant family, followed by *Flavobacteriaceae*, *Cryomorphaceae* and *Alteromonadaceae* (Table 1, Supplementary 4). Moreover, the presence of OTUs commonly associated with fecal wastes and secondary treatment of waste-water in WWTPs (*Thiothrix*, *Zoogloea*, *Enterobacteriaceae*, *Clostridium* or *Ruminococcus*; Supplementary 6) highlighted anthropogenic pressures (population density, industries, WWTP discharges) in this estuary. These results indicate that the water control policies of this UNESCO biosphere reserve have to be improved, in order to reduce the presence of these bacteria and that 16S amplicon sequencing is a valuable approach to detect unexpected waste discharges. Moreover, we found several bacterial taxa involved in the turnover of organic carbon and the cycling of nitrogen and sulfur, confirming the role of prokaryotes in biochemical processes [58]. This is due to the influence

of resuspension in sandy bottom systems, whereby the bacterial community of the sediments appears to get resuspended with each tide cycle, when the mass of seawater enters into the estuary stirring the matter of the bottom [59]. The influence of resuspension in sandy bottom systems has already been evidenced for meiobenthonic copepods, eukaryotic microorganisms, bacteria and viruses [29, 60]. This event would mainly take place in the inner water mass of the Urdaibai estuary (U30 and U33) and would explain the bacterial composition similarities of those two water masses along the annual cycle (not-significant ANOSIM values, Table 2, Fig. 3). Besides the resuspension, the existence of stagnant waters in the inner waters could also explain the presence of a distinct bacterial community. Despite the estuary empties with each tide, a high water residence time for stagnant waters has been described in the inner zone of Urdaibai [33, 34]. In these stagnant waters, several planktonic taxa have been shown to attain bloom proportions during periods of stable weather and low river discharge [33, 34].

Focusing on the community differences between water masses, *Betaproteobacteria* members, particularly *Comamonadaceae* (Table 2) and *Rhodocyclaceae*, together with *Bacteroidales* or *Microbacteriaceae* were more abundant in the inner water samples (Table 1, Supplementary 4). Differences in abundance of *Comamonadaceae* have been ascribed to terrestrial influence, as Newton and colleagues (2013) [61] described in Mississippi water samples. Interestingly, a longitudinal physicochemical gradient has been reported for Urdaibai estuary [62], whereby decreasing concentrations of nutrients (nitrites, nitrates, phosphates, DOC, etc.) have been recorded from inner to outer locations. This could explain the higher abundances of *Microbacteriaceae* in the inner zone, as this family has been associated to niches of high nutrient availability [56]. Similarly, taxa previously shown to be adapted to the oligotrophic oceanic ecosystems [56, 63–66], such as those affiliated within the *Pelagibacteraceae*, *Halomonadaceae*, *Synechococcaceae*, *SAR406* and Marine group II (Euryarchaeota) were significantly more abundant in outer samples, U35 (Table 1, Supplementary 4), reflecting coastal waters.

Seasonal changes were also observed for the bacteria composition (Fig. 2). The differences for each period were defined by the changes in the abundances of different bacterial groups. For example, *Pseudomonadales*, *Bacteroidales* and *Campylobacteraceae* showed the highest abundances during the cold period, while these groups of bacteria had a low abundance during the warm period (Table 1). *Flavobacteriales* and *Rhodobacteraceae* showed an abundance peak in spring in all water masses (Supplementary 5). *Rhodobacteraceae* spring bloom was previously described in a 6-year time series of Plymouth´s L4 station (western English Channel) analysed by Gilbert and colleagues [67]. It is to be highlighted that those two groups, *Flavobacteriales* and *Rhodobacteraceae*, comprised more than 40% of the bacterial community in spring samples for all water masses. This spring peak coincides with a decrease in alpha diversity and evenness (Supplementary 5), which suggests that the abundant OTUs might have outshined the rare OTUs limiting our ability to detect low abundant organisms through Illumina amplicon technique [68]. On the contrary, an increase of the bacterioplankton alpha diversity was detected in the cold period (fall-winter) in U35 samples (Supplementary 5). The high alpha diversity values coincide with a low total bacterial density (counts by microscopy), which could be attributable to the selective predation on most abundant bacterial groups, and it would explain the higher population evenness observed for this period and water mass. Under this scenario, the amplicon sequencing method was able to detect higher number of low abundant taxa.

Regarding bacterial count changes along the annual cycle in U35, similar results of those observed in the present study were obtained by Huete-Stauffer & Morán [69] in a time-series (2009-2011) study in the continental stations of Xixón (Spain), located also in the Bay of Biscay coastal waters. These variations could be related with temperature changes, as it is known that low temperature slows down bacterial metabolism [69, 70], while an increase of temperature stimulates both bacterial growth and metabolism [71, 72], coinciding with the abundance patterns observed in this study. Predatory and lythic activities that control bacterial communities are affected by temperature [31, 73, 74], which control bacterial communities. Thus, the bacterial abundance decline observed du-

ring the “warm season” in the Urdaibai estuary could be related to the typical plankton cycle whereby after the first primary producers peak of spring, there is a second peak of heterotrophic organisms that feed on them [31], coinciding with the total bacteria abundance decline observed in May-June months in this study.

Conclusions

In conclusion, regardless of the reasons for their origin -anthropogenic impact (WWTP, industry, etc.), terrestrial effect, resuspension of the riverbed, stagnant waters- bacterial communities from inner and outer waters are differentiated in drainage systems, as it happens in stratified estuaries. Demonstrating a high bacterial community complexity in drainage estuaries, as well as that amplicon sequencing is a valuable technique for tracking bacterial community changes and their temporal monitoring. The use of the information generated with such method would allow a more effective implementation of water control policies, especially valuable for protected areas such as Urdaibai, a biosphere reserve.

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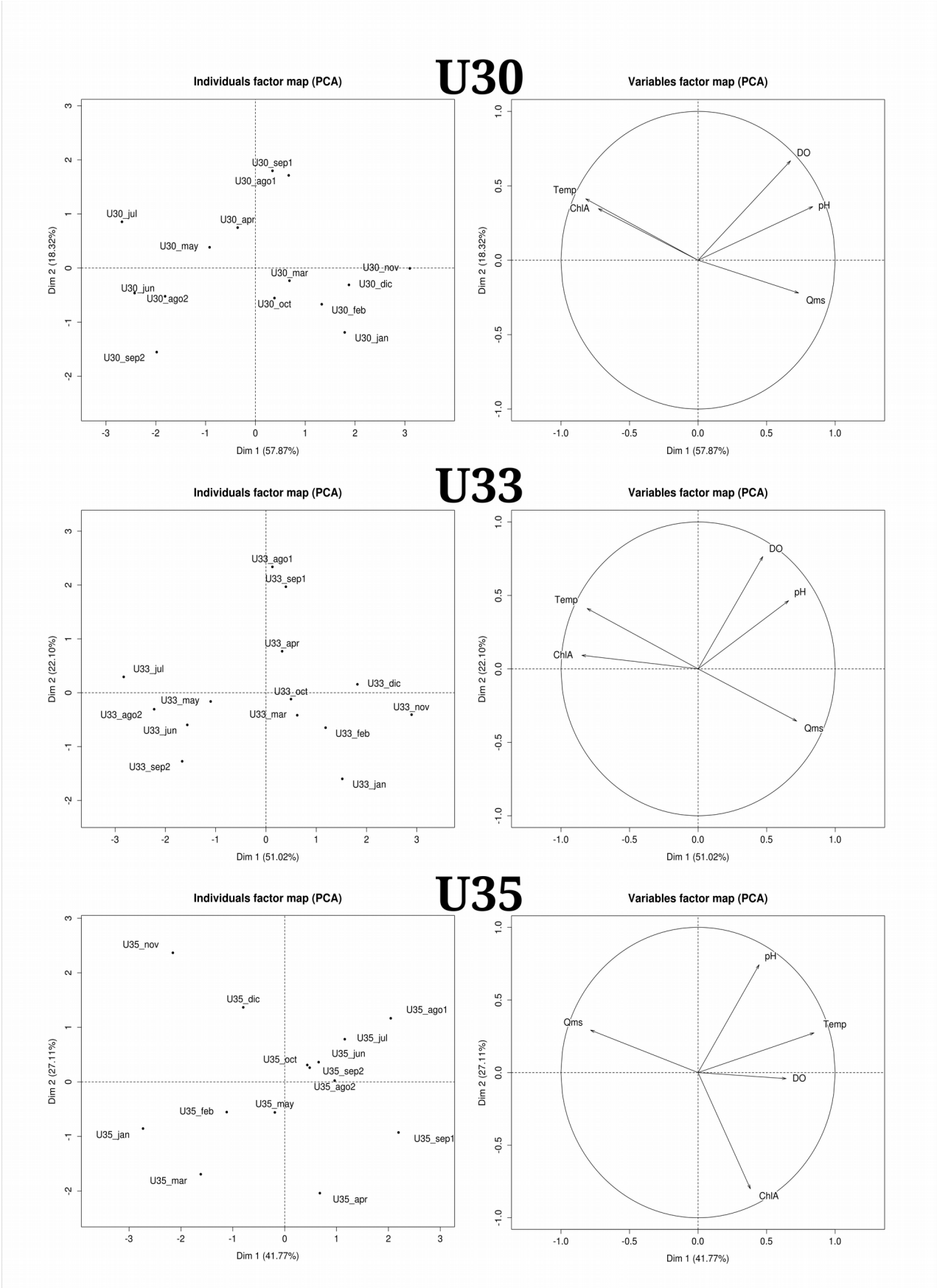
Supplementary material

Supplementary 1: PCA of physicochemical variables of the water masses. On the left, PCA plots where the samples grouped by the environmental variables distribution. In other words, the samples of summer, with higher temperatures, are grouped in the same area of the direction of the temperature arrow, of the variable factor map (individual factor map). On the right, PCA plots with the environmental variables correlation (Variables factor map).


U30
Individuals factor map (PCA)
U30_sep1
U30_ago1
U30_jul
U30_apr
U30_may
U30_nov
U30_mar
U30_jun
U30_dic
U30_ago2
U30_oct
U30_feb
U30_jan
U30_sep2
Dim 2 (18.32%)
Dim 1 (57.87%)
Variables factor map (PCA)
DO
Temp
ChlA
pH
Qms
Dim 2 (18.32%)
Dim 1 (57.87%)
U33
Individuals factor map (PCA)
U33_ago1
U33_sep1
U33_apr
U33_jul
U33_dic
U33_oct
U33_may
U33_nov
U33_ago2
U33_mar
U33_jun
U33_feb
U33_sep2
U33_jan
Dim 2 (22.10%)
Dim 1 (51.02%)
Variables factor map (PCA)
DO
pH
Temp
ChlA
Qms
Dim 2 (22.10%)
Dim 1 (51.02%)
U35
Individuals factor map (PCA)
U35_nov
U35_dic
U35_ago1
U35_jul
U35_jun
U35_oct
U35_sep2
U35_ago2
U35_may
U35_feb
U35_jan
U35_sep1
U35_mar
U35_apr
Dim 2 (27.11%)
Dim 1 (41.77%)
Variables factor map (PCA)
pH
Qms
Temp
DO
ChlA
Dim 2 (27.11%)
Dim 1 (41.77%)

Supplementary 2: Spearman's correlation between the environmental variables and OTUs for each water mass. Spearman's correlation between the environmental variables and OTUs for each water mass: temperature (Temp), river discharge (Qms), dissolved oxygen (DO), chlorophyll (ChlA) and pH. The within environmental variables correlation and the cumulative correlations are shown (sorted in descending order of significance).

U30

Variable	correlation	Variable cumulative	correlation cumulative
Temp	0.80	Temp	0.80
Qms	0.80	Qms, Temp	0.85
ChlA	0.29	Qms, ChlA, Temp	0.77
pH	0.28	Qms, ChlA, Temp, pH	0.70
DO	0.22	Qms, ChlA, Temp, DO, pH	0.64

U33

Variable	correlation	Variable cumulative	correlation cumulative
Temp	0.75	Temp	0.75
Qms	0.72	Qms, Temp	0.78
DO	0.25	Qms, ChlA, Temp	0.66
ChlA	0.23	Qms, ChlA, Temp, pH	0.58
pH	0.05	Qms, ChlA, Temp, DO, pH	0.52

U35

Variable	correlation	Variable cumulative	correlation cumulative
Qms	0.54	Qms	0.54
Temp	0.52	Qms, Temp	0.57
pH	0.31	Qms, ChlA, Temp	0.56
ChlA	0.15	Qms, ChlA, Temp, pH	0.55
DO	-0.05	Qms, ChlA, Temp, DO, pH	0.41

Supplementary 3: Bacterial OTUs significantly correlated with temperature. The most significant OTUs showing negative or positive Spearman correlations with temperature (rho value > 0.7).

OTU	Spearman value	Assigned taxonomy
OTUs1	-0.8843536455	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
OTUs2	-0.8412170533	p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Aeromonadaceae
OTUs3	-0.8281195212	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs4	-0.822159055	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter
OTUs5	-0.8000114995	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs6	-0.7918175597	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas
OTUs7	-0.7875027746	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
OTUs8	-0.7873664444	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
OTUs9	-0.7813253728	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs10	-0.7786182763	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
OTUs11	-0.7782683879	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
OTUs12	-0.7779100585	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;g_Fluviicola
OTUs13	-0.7724068066	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs14	-0.770115408	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs15	-0.7675078079	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Rhodobacter
OTUs16	-0.7661935833	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs17	-0.7627427518	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs18	-0.7626224553	p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales
OTUs19	-0.7622494162	p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae
OTUs20	-0.7602794364	p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales
OTUs21	-0.7568788658	p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae
OTUs22	-0.7564559213	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
OTUs23	-0.7555732209	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs24	-0.7469716492	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs25	-0.7463040206	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs26	-0.7438421782	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae;g_CandidatusPortiera
OTUs27	-0.7402632611	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs28	-0.7400167108	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs29	-0.7399074419	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs30	-0.734616708	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides
OTUs31	-0.734262231	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs32	-0.7337187543	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter
OTUs33	-0.7336979201	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
OTUs34	-0.7328517375	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs35	-0.7304862276	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs36	-0.7289275521	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs37	-0.7184078862	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs38	-0.7180726417	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Citrobacter
OTUs39	-0.7160817006	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs40	-0.7159783137	p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Propionivibrio
OTUs41	-0.7159119122	p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae
OTUs42	-0.7156980602	p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;NA;NA
OTUs43	-0.7142148648	p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Succinivibrionaceae;g_Succinivibrio
OTUs44	-0.7132622938	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Sulfurospirillum
OTUs45	-0.713146963	p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_uniformis
OTUs46	-0.7104602909	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas
OTUs47	-0.7098146313	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_[Chromatiaceae];g_Rheinheimera
OTUs48	-0.7097827539	p_SAR406;c_AB16;o_Arctic96B-7;f_A714017;g_SargSea-WGS
OTUs49	-0.7090459641	p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;f_Methylophilaceae;g_Methylotenera;s_mobilis
OTUs50	-0.7084024633	p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
OTUs51	-0.7080202257	p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas
OTUs52	-0.7062697211	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs53	-0.7056195919	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Tenacibaculum
OTUs54	-0.7052699197	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs55	-0.7035233122	p_Proteobacteria;c_Alphaproteobacteria
OTUs56	-0.7033454872	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Marinomonas
OTUs57	-0.7017800803	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
OTUs58	0.701350335	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs59	0.7016663986	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
OTUs60	0.7020753886	p_Proteobacteria;c_Alphaproteobacteria;o_Kiloniellales;f_Kiloniellaceae
OTUs61	0.7062273644	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
OTUs62	0.708381162	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae

OTUs63	0.709138515	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs64	0.7113496441	p_Proteobacteria;c_Deltaproteobacteria;o_Myxococcales;f_OM27
OTUs65	0.7125941245	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
OTUs66	0.712925674	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
OTUs67	0.7135260054	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs68	0.7138178396	p_Proteobacteria;c_Alphaproteobacteria
OTUs69	0.7144752923	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
OTUs70	0.7153025163	Unassigned;NA;NA;NA;NA;NA;NA
OTUs71	0.7154450372	p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_NS11-12
OTUs72	0.7174386385	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
OTUs73	0.7185000676	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;NA;NA
OTUs74	0.7192702742	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_NS9
OTUs75	0.7207550765	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;NA;NA
OTUs76	0.7208970069	p_Proteobacteria;c_Gammaproteobacteria;o_HTCC2188
OTUs77	0.7255138967	p_Proteobacteria;c_Alphaproteobacteria
OTUs78	0.7273303626	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;NA;NA
OTUs79	0.7284774562	p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;f_Methylophilaceae
OTUs80	0.7285892104	p_Proteobacteria;c_Gammaproteobacteria;o_HTCC2188
OTUs81	0.7297052334	p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae
OTUs82	0.7313422538	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;NA;NA
OTUs83	0.7314922827	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
OTUs84	0.7316701642	p_Verrucomicrobia;c_Opitutae;o_Opitutales;f_Opitutaceae;g_Opitutus
OTUs85	0.735391607	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs86	0.7366185227	p_Proteobacteria;c_Alphaproteobacteria
OTUs87	0.7368773524	Unassigned;NA;NA;NA;NA;NA;NA
OTUs88	0.7403579198	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;NA;NA
OTUs89	0.7448875861	p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_C111
OTUs90	0.7456742818	p_Proteobacteria;c_Deltaproteobacteria;o_Myxococcales;f_OM27
OTUs91	0.7461203922	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;g_Fluviicola
OTUs92	0.7465563727	p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales
OTUs93	0.7466019525	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs94	0.7476008371	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
OTUs95	0.7482676411	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae
OTUs96	0.748350786	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
OTUs97	0.7487978165	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
OTUs98	0.7529056637	p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales
OTUs99	0.7553251443	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
OTUs100	0.7555877857	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs101	0.7593492143	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs102	0.7604186259	p_Proteobacteria;c_Alphaproteobacteria
OTUs103	0.760922889	p_Tenericutes;c_Mollicutes;o_Acholeplasmatales;f_Acholeplasmataceae;g_Acholeplasma
OTUs104	0.7630621898	p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae
OTUs105	0.7666044129	p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_CandidatusAquiluna;s_rubra
OTUs106	0.7685055352	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs107	0.7698861238	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs108	0.7702130964	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs109	0.772783275	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;NA;NA
OTUs110	0.7759031116	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
OTUs111	0.7781941564	p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae
OTUs112	0.7854689166	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs113	0.7864979871	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
OTUs114	0.7869547866	p_Bacteroidetes;c_[Saprospirae];o_[Saprospirales];f_Saprospiraceae
OTUs115	0.7895351133	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
OTUs116	0.7912641156	p_Proteobacteria;c_Alphaproteobacteria
OTUs117	0.7918928436	p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_CandidatusAquiluna;s_rubra
OTUs118	0.7925933409	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
OTUs119	0.7926296344	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
OTUs120	0.7951203033	p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales
OTUs121	0.7964555946	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs122	0.7969535174	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs123	0.7978455936	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
OTUs124	0.7982235496	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
OTUs125	0.7985711698	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
OTUs126	0.7999069936	p_Proteobacteria;c_Alphaproteobacteria
OTUs127	0.7999937442	p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales
OTUs128	0.801504404	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs129	0.80501808	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
OTUs130	0.8108433264	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs131	0.8121624164	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;g_Crocinitomix
OTUs132	0.8166970584	p_Bacteroidetes;c_[Rhodothermi];o_[Rhodothermales];f_[Balneolaceae];g_Balneola
OTUs133	0.8200345869	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs134	0.8216382678	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae

OTUs135	0.8218428828	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
OTUs136	0.8225605281	p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
OTUs137	0.8406194217	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs138	0.8409746248	p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
OTUs139	0.8501458064	p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
OTUs140	0.8551633639	p_Proteobacteria;c_Gammaproteobacteria;o_HTCC2188
OTUs141	0.8650183274	p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
OTUs142	0.8990004487	Unassigned;NA;NA;NA;NA;NA;NA

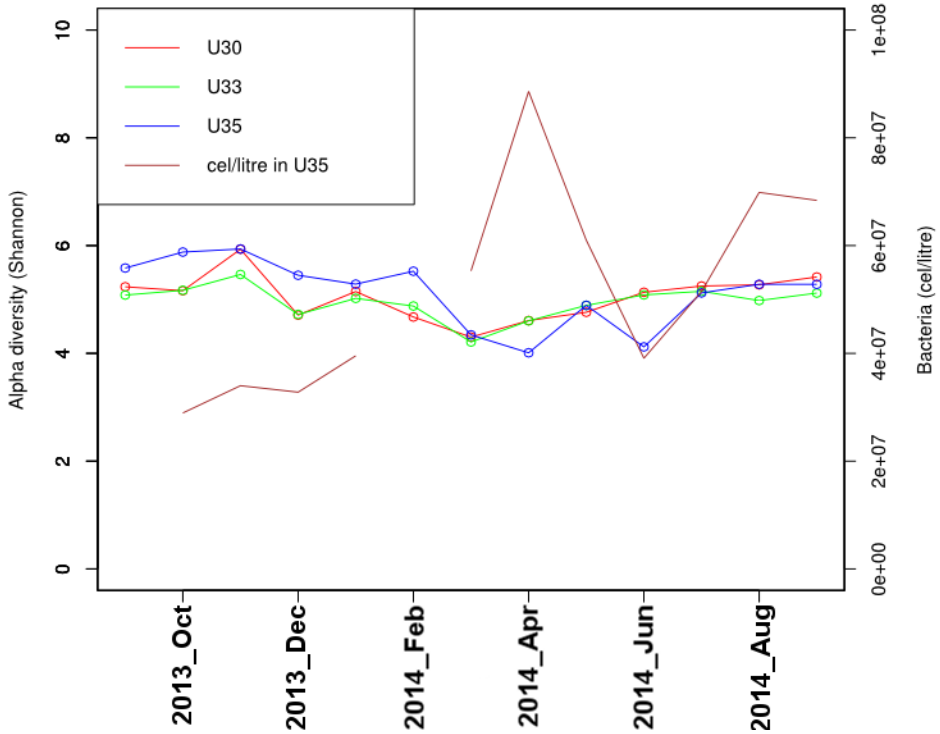
Supplementary 4: Community abundance variations long the annual cycle per estuarine water mass. The percentages for each taxonomy group were shown. Only the bacterial groups that at some point reach 1% of the community are shown.

U30 Kingdom	Phylum	Class	Order	Family	2013_Sep	2013_Oct	2013_Nov	2013_Dec	2014_Ene	2014_Feb	2014_Mar	2014_Abr	2014_May	2014_Jun	2014_Jul	2014_Ago	2014_Sep	Anual average
Archaea					0.005	0.008	0.027	0.011	0.020	0.008	0.008	0.006	0.003	0.004	0.002	0.010	0.002	0.86 %
	Euryarchaeota				0.005	0.006	0.019	0.008	0.010	0.004	0.008	0.006	0.003	0.003	0.001	0.010	0.002	0.65 %
		Thermoplasmata			0.005	0.006	0.019	0.008	0.010	0.004	0.008	0.006	0.003	0.003	0.001	0.010	0.002	0.65 %
			E2		0.005	0.006	0.019	0.008	0.010	0.004	0.008	0.006	0.003	0.003	0.001	0.010	0.002	0.65 %
				Marine group II	0.005	0.006	0.017	0.008	0.009	0.004	0.008	0.005	0.003	0.003	0.001	0.010	0.002	0.62 %
Bacteria					0.970	0.982	0.961	0.975	0.972	0.978	0.970	0.963	0.960	0.973	0.953	0.960	0.976	96.87 %
	Proteobacteria				0.656	0.665	0.646	0.691	0.689	0.701	0.682	0.640	0.647	0.616	0.644	0.676	0.663	66.30 %
		Alphaproteobacteria			0.308	0.328	0.222	0.207	0.240	0.209	0.286	0.327	0.302	0.291	0.342	0.315	0.304	28.32 %
			Rhodobacterales		0.205	0.225	0.119	0.155	0.159	0.166	0.230	0.254	0.236	0.202	0.216	0.198	0.195	19.70 %
				Rhodobacteraceae	0.203	0.220	0.118	0.154	0.155	0.164	0.227	0.253	0.232	0.200	0.211	0.197	0.193	19.42 %
			Rickettsiales		0.029	0.032	0.033	0.022	0.024	0.011	0.013	0.018	0.013	0.022	0.033	0.036	0.035	2.47 %
				Pelagibacteraceae	0.016	0.016	0.019	0.014	0.018	0.008	0.012	0.016	0.011	0.016	0.027	0.030	0.024	1.76 %
		Gammaproteobacteria			0.233	0.229	0.261	0.273	0.286	0.282	0.211	0.171	0.193	0.211	0.209	0.246	0.233	23.37 %
			Alteromonadales		0.109	0.107	0.082	0.115	0.090	0.114	0.123	0.115	0.118	0.106	0.103	0.110	0.112	10.79 %
				Alteromonadaceae	0.039	0.044	0.027	0.052	0.041	0.054	0.062	0.038	0.042	0.035	0.027	0.030	0.033	4.02 %
				HTCC2188	0.026	0.025	0.017	0.040	0.015	0.035	0.037	0.045	0.037	0.025	0.029	0.026	0.030	2.98 %
				OM60	0.039	0.030	0.021	0.011	0.014	0.011	0.017	0.029	0.033	0.040	0.039	0.047	0.040	2.86 %
			Oceanospirillales		0.062	0.066	0.060	0.053	0.095	0.046	0.024	0.017	0.042	0.043	0.049	0.072	0.054	5.25 %
				Oceanospirillaceae	0.020	0.022	0.019	0.030	0.047	0.020	0.008	0.007	0.007	0.011	0.016	0.034	0.019	2.00 %
				Halomonadaceae	0.020	0.023	0.026	0.015	0.031	0.016	0.004	0.005	0.015	0.013	0.012	0.018	0.013	1.62 %
			Vibrionales		0.005	0.008	0.009	0.006	0.012	0.005	0.006	0.006	0.005	0.007	0.007	0.010	0.011	0.75 %
			Chromatiales		0.016	0.012	0.016	0.009	0.012	0.011	0.006	0.006	0.007	0.019	0.018	0.023	0.020	1.34 %
			Thiotrichales		0.009	0.007	0.021	0.010	0.015	0.009	0.006	0.005	0.005	0.008	0.006	0.005	0.008	0.87 %
				Piscirickettsiaceae	0.007	0.006	0.016	0.009	0.013	0.006	0.005	0.004	0.004	0.006	0.005	0.004	0.006	0.70 %
			Pseudomonadales		0.005	0.004	0.025	0.031	0.021	0.036	0.014	0.007	0.005	0.006	0.001	0.002	0.004	1.24 %
		Betaproteobacteria			0.069	0.066	0.071	0.130	0.066	0.130	0.138	0.118	0.114	0.074	0.059	0.065	0.065	8.96 %
			Burkholderiales		0.034	0.035	0.037	0.077	0.038	0.080	0.087	0.079	0.069	0.041	0.028	0.034	0.039	5.22 %
				Comamonadaceae	0.032	0.032	0.033	0.072	0.034	0.075	0.080	0.076	0.066	0.040	0.026	0.031	0.034	4.86 %
			Methylophilales		0.018	0.018	0.009	0.016	0.008	0.013	0.021	0.020	0.024	0.016	0.015	0.015	0.010	1.55 %
				Methylophilaceae	0.018	0.018	0.009	0.016	0.008	0.013	0.021	0.020	0.024	0.016	0.015	0.015	0.010	1.54 %
			Rhodocyclales		0.007	0.007	0.014	0.026	0.013	0.027	0.023	0.014	0.015	0.008	0.006	0.004	0.007	1.31 %
				Rhodocyclaceae	0.007	0.007	0.014	0.026	0.013	0.027	0.023	0.014	0.015	0.008	0.006	0.004	0.007	1.31 %
		Epsilonproteobacteria			0.023	0.023	0.040	0.057	0.071	0.053	0.034	0.016	0.020	0.016	0.007	0.020	0.026	3.13 %
			Campylobacterales		0.023	0.023	0.040	0.057	0.071	0.053	0.034	0.016	0.020	0.016	0.007	0.020	0.026	3.13 %
				Campylobacteraceae	0.019	0.017	0.029	0.050	0.062	0.044	0.029	0.014	0.015	0.012	0.004	0.016	0.021	2.57 %
		Deltaproteobacteria			0.022	0.020	0.053	0.024	0.025	0.027	0.013	0.007	0.019	0.023	0.027	0.029	0.035	2.49 %
	Bacteroidetes				0.217	0.230	0.180	0.188	0.184	0.205	0.228	0.251	0.232	0.249	0.208	0.188	0.217	21.36 %
		Flavobacteriia			0.190	0.204	0.115	0.114	0.134	0.134	0.180	0.216	0.200	0.219	0.184	0.164	0.181	17.20 %
			Flavobacteriales		0.190	0.204	0.115	0.114	0.134	0.134	0.180	0.216	0.200	0.219	0.184	0.164	0.181	17.20 %
				Flavobacteriaceae	0.105	0.118	0.056	0.057	0.072	0.071	0.095	0.115	0.098	0.104	0.084	0.086	0.083	8.80 %
				Cryomorphaceae	0.067	0.063	0.042	0.046	0.048	0.054	0.073	0.090	0.075	0.086	0.085	0.065	0.081	6.75 %
		Bacteroidia			0.013	0.012	0.043	0.063	0.036	0.053	0.022	0.011	0.011	0.011	0.005	0.006	0.011	2.27 %
			Bacteroidales		0.013	0.012	0.043	0.063	0.036	0.053	0.022	0.011	0.011	0.011	0.005	0.006	0.011	2.27 %
	Actinobacteria				0.066	0.053	0.028	0.027	0.024	0.013	0.034	0.057	0.063	0.077	0.069	0.064	0.060	4.90 %
		Actinobacteria			0.053	0.039	0.007	0.010	0.005	0.005	0.030	0.052	0.059	0.070	0.062	0.058	0.053	3.87 %
			Actinomycetales		0.053	0.039	0.006	0.009	0.004	0.004	0.029	0.052	0.059	0.070	0.062	0.058	0.052	3.83 %
				Microbacteriaceae	0.052	0.037	0.004	0.008	0.003	0.003	0.027	0.050	0.058	0.067	0.058	0.053	0.048	3.61 %
		Acidimicrobiia			0.013	0.014	0.022	0.017	0.019	0.008	0.005	0.005	0.004	0.007	0.006	0.006	0.007	1.01 %
			Acidimicrobiales		0.013	0.014	0.022	0.017	0.019	0.008	0.005	0.005	0.004	0.007	0.006	0.006	0.007	1.01 %
				OCS155	0.011	0.011	0.014	0.012	0.012	0.005	0.004	0.004	0.004	0.004	0.003	0.004	0.005	0.72 %
	Verrucomicrobia				0.013	0.005	0.013	0.006	0.007	0.007	0.004	0.002	0.005	0.012	0.018	0.011	0.016	0.92 %
	Cyanobacteria				0.005	0.009	0.008	0.004	0.004	0.003	0.002	0.001	0.003	0.004	0.005	0.004	0.004	0.42 %
		c_Synechococcophycideae			0.005	0.009	0.007	0.003	0.004	0.002	0.001	0.001	0.002	0.003	0.005	0.004	0.004	0.38 %
			Synechococcales		0.005	0.009	0.007	0.003	0.004	0.002	0.001	0.001	0.002	0.003	0.005	0.004	0.004	0.38 %
				Synechococcaceae	0.005	0.009	0.007	0.003	0.004	0.002	0.001	0.001	0.002	0.003	0.005	0.004	0.004	0.38 %
	SAR406				0.003	0.006	0.020	0.007	0.010	0.003	0.001	0.000	0.000	0.001	0.000	0.003	0.002	0.44 %
		c_AB16			0.003	0.006	0.020	0.007	0.010	0.003	0.001	0.000	0.000	0.001	0.000	0.003	0.002	0.44 %

U33 Kingdom	Phylum	Class	Order	Family	2013_Sep	2013_Oct	2013_Nov	2013_Dec	2014_Ene	2014_Feb	2014_Mar	2014_Abr	2014_May	2014_Jun	2014_Jul	2014_Ago	2014_Sep	Anual average
Archaea					0.011	0.003	0.026	0.017	0.015	0.013	0.013	0.011	0.003	0.007	0.003	0.013	0.005	1.06 %
	Euryarchaeota				0.010	0.003	0.017	0.014	0.008	0.007	0.012	0.010	0.003	0.006	0.002	0.012	0.005	0.83 %
		Thermoplasmata			0.010	0.003	0.017	0.014	0.008	0.007	0.012	0.010	0.003	0.006	0.002	0.012	0.005	0.83 %
			E2		0.010	0.003	0.017	0.014	0.008	0.007	0.012	0.010	0.003	0.006	0.002	0.012	0.005	0.83 %
				Marine group II	0.010	0.002	0.015	0.013	0.008	0.007	0.012	0.010	0.003	0.006	0.002	0.012	0.005	0.80 %
Bacteria					0.971	0.985	0.960	0.969	0.976	0.971	0.966	0.961	0.969	0.966	0.960	0.957	0.974	96.79 %
	Proteobacteria				0.644	0.670	0.661	0.704	0.678	0.680	0.677	0.640	0.650	0.626	0.654	0.656	0.648	66.07 %
		Alphaproteobacteria			0.339	0.306	0.230	0.208	0.232	0.201	0.301	0.337	0.303	0.324	0.367	0.351	0.345	29.59 %
			Rhodobacterales		0.217	0.205	0.130	0.156	0.173	0.147	0.219	0.245	0.229	0.218	0.238	0.228	0.221	20.20 %
				Rhodobacteraceae	0.215	0.200	0.128	0.155	0.171	0.141	0.214	0.241	0.225	0.215	0.234	0.227	0.219	19.89 %
			Rickettsiales		0.037	0.030	0.033	0.020	0.015	0.017	0.025	0.027	0.015	0.028	0.035	0.040	0.043	2.81 %
				Pelagibacteraceae	0.021	0.013	0.021	0.014	0.010	0.011	0.019	0.025	0.013	0.024	0.029	0.035	0.031	2.04 %
		Gammaproteobacteria			0.231	0.231	0.265	0.287	0.245	0.285	0.235	0.193	0.204	0.203	0.216	0.226	0.220	23.38 %
			Alteromonadales		0.103	0.123	0.100	0.141	0.097	0.135	0.135	0.126	0.122	0.107	0.104	0.104	0.113	11.62 %
				Alteromonadaceae	0.040	0.047	0.032	0.056	0.037	0.051	0.055	0.046	0.042	0.037	0.034	0.033	0.039	4.22 %
				HTCC2188	0.022	0.029	0.019	0.037	0.010	0.026	0.035	0.041	0.037	0.023	0.021	0.018	0.026	2.64 %
				OM60	0.037	0.033	0.016	0.014	0.013	0.014	0.026	0.032	0.031	0.039	0.043	0.047	0.041	2.97 %
			Oceanospirillales		0.077	0.057	0.057	0.062	0.068	0.051	0.043	0.023	0.042	0.045	0.059	0.071	0.057	5.49 %
				Oceanospirillaceae	0.025	0.021	0.017	0.033	0.038	0.016	0.022	0.011	0.011	0.008	0.017	0.031	0.021	2.09 %
				Halomonadaceae	0.031	0.013	0.026	0.018	0.018	0.018	0.007	0.005	0.016	0.016	0.020	0.023	0.018	1.76 %
			Vibrionales		0.006	0.007	0.008	0.008	0.012	0.008	0.010	0.006	0.005	0.006	0.006	0.006	0.005	0.72 %
			Chromatiales		0.011	0.013	0.015	0.010	0.009	0.013	0.007	0.008	0.008	0.015	0.014	0.017	0.015	1.20 %
			Thiotrichales		0.008	0.006	0.017	0.011	0.010	0.013	0.008	0.006	0.005	0.008	0.007	0.008	0.007	0.88 %
				Piscirickettsiaceae	0.008	0.005	0.015	0.009	0.008	0.010	0.005	0.005	0.005	0.007	0.006	0.007	0.006	0.74 %
			Pseudomonadales		0.002	0.003	0.027	0.027	0.020	0.029	0.013	0.009	0.007	0.003	0.000	0.001	0.000	1.08 %
		Betaproteobacteria			0.048	0.078	0.079	0.132	0.088	0.111	0.102	0.093	0.100	0.064	0.043	0.038	0.049	7.86 %
			Burkholderiales		0.017	0.044	0.047	0.077	0.053	0.067	0.065	0.057	0.060	0.031	0.020	0.014	0.025	4.45 %
				Comamonadaceae	0.016	0.042	0.041	0.071	0.048	0.063	0.060	0.055	0.057	0.030	0.018	0.013	0.023	4.12 %
			Methylophilales		0.018	0.017	0.008	0.016	0.008	0.012	0.018	0.019	0.021	0.018	0.012	0.010	0.013	1.46 %
				Methylophilaceae	0.018	0.017	0.007	0.015	0.008	0.012	0.018	0.019	0.021	0.018	0.012	0.010	0.013	1.45 %
			Rhodocyclales		0.004	0.009	0.014	0.026	0.018	0.023	0.015	0.012	0.013	0.006	0.003	0.003	0.004	1.15 %
				Rhodocyclaceae	0.004	0.009	0.014	0.026	0.018	0.023	0.015	0.012	0.013	0.006	0.003	0.003	0.004	1.15 %
		Epsilonproteobacteria			0.013	0.033	0.041	0.054	0.085	0.048	0.027	0.013	0.022	0.012	0.007	0.011	0.013	2.90 %
			Campylobacterales		0.013	0.033	0.041	0.054	0.085	0.048	0.027	0.013	0.022	0.012	0.007	0.011	0.013	2.90 %
				Campylobacteraceae	0.010	0.026	0.032	0.046	0.074	0.039	0.024	0.010	0.018	0.008	0.004	0.007	0.010	2.37 %
		Deltaproteobacteria			0.013	0.022	0.046	0.024	0.028	0.036	0.011	0.005	0.020	0.023	0.021	0.030	0.022	2.32 %
	Bacteroidetes				0.233	0.215	0.166	0.173	0.197	0.213	0.241	0.247	0.239	0.239	0.208	0.212	0.233	21.67 %
		Flavobacteriia			0.217	0.179	0.113	0.110	0.138	0.139	0.194	0.220	0.208	0.215	0.186	0.187	0.209	17.80 %
			Flavobacteriales		0.217	0.179	0.113	0.110	0.138	0.139	0.194	0.220	0.208	0.215	0.186	0.187	0.209	17.80 %
				Flavobacteriaceae	0.129	0.105	0.052	0.057	0.071	0.076	0.111	0.117	0.103	0.098	0.093	0.095	0.107	9.33 %
				Cryomorphaceae	0.069	0.056	0.045	0.043	0.056	0.054	0.066	0.087	0.080	0.089	0.083	0.078	0.083	6.82 %
		Bacteroidia			0.003	0.023	0.036	0.052	0.042	0.050	0.015	0.006	0.012	0.009	0.005	0.004	0.004	2.02 %
			Bacteroidales		0.003	0.023	0.036	0.052	0.042	0.050	0.015	0.006	0.012	0.009	0.005	0.004	0.004	2.02 %
	Actinobacteria				0.068	0.050	0.010	0.010	0.009	0.004	0.022	0.050	0.050	0.061	0.060	0.056	0.059	3.92 %
		Actinobacteria			0.051	0.050	0.010	0.010	0.009	0.004	0.022	0.050	0.050	0.061	0.053	0.047	0.047	3.57 %
			Actinomycetales		0.051	0.049	0.009	0.009	0.007	0.004	0.022	0.050	0.050	0.061	0.053	0.047	0.047	3.53 %
				Microbacteriaceae	0.049	0.047	0.007	0.007	0.006	0.003	0.020	0.048	0.049	0.058	0.048	0.044	0.044	3.31 %
		Acidimicrobiia			0.017	0.010	0.022	0.015	0.013	0.011	0.006	0.008	0.003	0.008	0.008	0.008	0.011	1.09 %
			Acidimicrobiales		0.017	0.010	0.022	0.015	0.013	0.011	0.006	0.008	0.003	0.008	0.008	0.008	0.011	1.09 %
				OCS155	0.015	0.005	0.012	0.006	0.007	0.006	0.005	0.003	0.004	0.014	0.005	0.007	0.010	0.75 %
	Verrucomicrobia				0.006	0.005	0.012	0.006	0.007	0.006	0.005	0.003	0.004	0.014	0.015	0.010	0.014	0.81 %
	Cyanobacteria				0.005	0.007	0.008	0.006	0.004	0.002	0.002	0.002	0.007	0.006	0.014	0.009	0.009	0.63 %
		c_Synechococcophycideae			0.005	0.006	0.007	0.006	0.003	0.002	0.002	0.002	0.007	0.006	0.014	0.008	0.009	0.59 %
			Synechococcales		0.005	0.006	0.007	0.006	0.003	0.002	0.002	0.002	0.007	0.006	0.014	0.008	0.009	0.59 %
				Synechococcaceae	0.005	0.003	0.019	0.009	0.006	0.004	0.002	0.002	0.000	0.000	0.014	0.008	0.009	0.64 %
	SAR406				0.006	0.003	0.019	0.009	0.006	0.004	0.002	0.002	0.000	0.000	0.001	0.004	0.005	0.48 %
		c_AB16			0.006	0.003	0.019	0.009	0.006	0.004	0.002	0.002	0.000	0.000	0.001	0.004	0.005	0.48 %

U35 Kingdom	Phylum	Class	Order	Family	2013_Sep	2013_Oct	2013_Nov	2013_Dec	2014_Ene	2014_Feb	2014_Mar	2014_Abr	2014_May	2014_Jun	2014_Jul	2014_Ago	2014_Sep	Anual average
Archaea					0.018	0.025	0.046	0.036	0.020		0.016	0.002	0.009	0.007	0.004	0.012	0.011	1.71 %
	Euryarchaeota				0.016	0.021	0.029	0.028	0.011	0.014	0.015	0.002		0.006	0.003	0.011	0.011	1.40 %
		Thermoplasmata			0.016	0.021	0.029	0.028	0.011	0.014	0.015	0.002	0.009	0.006	0.003	0.011	0.011	1.36 %
			E2		0.016	0.021	0.029	0.028	0.011	0.014	0.015	0.002	0.009	0.006	0.003	0.011	0.011	1.36 %
				Marine group II	0.016	0.020	0.026	0.026	0.010	0.013	0.015	0.002	0.009	0.006	0.003	0.011	0.010	1.30 %
Bacteria					0.956	0.962	0.939	0.950	0.970	0.962	0.961	0.976	0.971	0.978	0.977	0.967	0.971	96.47 %
	Proteobacteria				0.623	0.635	0.625	0.655	0.704	0.684	0.685	0.744	0.665	0.715	0.641	0.671	0.651	66.91 %
		Alphaproteobacteria			0.304	0.267	0.274	0.220	0.250	0.244	0.381	0.429	0.338	0.331	0.342	0.326	0.334	31.09 %
			Rhodobacterales		0.170	0.142	0.102	0.115	0.172	0.146	0.238	0.258	0.224	0.198	0.170	0.188	0.191	17.79 %
				Rhodobacteraceae	0.168	0.135	0.098	0.112	0.167	0.135	0.231	0.254	0.218	0.196	0.161	0.186	0.187	17.29 %
			Rickettsiales		0.036	0.045	0.058	0.046	0.022	0.030	0.051	0.061	0.035	0.043	0.048	0.055	0.059	4.52 %
				Pelagibacteraceae	0.020	0.026	0.036	0.033	0.015	0.021	0.042	0.057	0.030	0.037	0.039	0.044	0.044	3.40 %
		Gammaproteobacteria			0.267	0.285	0.250	0.323	0.294	0.313	0.216	0.217	0.244	0.310	0.248	0.288	0.265	27.09 %
			Alteromonadales		0.099	0.108	0.071	0.110	0.099	0.114	0.112	0.114	0.121	0.116	0.097	0.118	0.104	10.63 %
				Alteromonadaceae	0.040	0.037	0.023	0.049	0.046	0.047	0.051	0.059	0.052	0.060	0.035	0.035	0.038	4.39 %
				HTCC2188	0.014	0.018	0.018	0.022	0.013	0.022	0.022	0.017	0.027	0.016	0.017	0.015	0.014	1.80 %
				OM60	0.030	0.034	0.018	0.020	0.017	0.024	0.023	0.037	0.033	0.032	0.035	0.037	0.033	2.88 %
			Oceanospirillales		0.089	0.085	0.081	0.118	0.093	0.077	0.051	0.056	0.075	0.119	0.086	0.109	0.094	8.72 %
				Oceanospirillaceae	0.030	0.027	0.014	0.062	0.048	0.021	0.030	0.025	0.028	0.065	0.023	0.053	0.044	3.61 %
				Halomonadaceae	0.041	0.040	0.048	0.040	0.027	0.036	0.005	0.020	0.035	0.034	0.035	0.035	0.037	3.33 %
			Vibrionales		0.020	0.013	0.013	0.014	0.014	0.013	0.019	0.012	0.013	0.035	0.021	0.025	0.034	1.89 %
			Chromatiales		0.007	0.011	0.012	0.010	0.013	0.019	0.002	0.005	0.004	0.006	0.003	0.006	0.005	0.80 %
			Thiotrichales		0.009	0.020	0.021	0.024	0.019	0.025	0.006	0.007	0.006	0.009	0.007	0.011	0.009	1.32 %
				Piscirickettsiaceae	0.009	0.017	0.020	0.020	0.014	0.019	0.005	0.007	0.004	0.008	0.007	0.009	0.008	1.13 %
			Pseudomonadales		0.001	0.003	0.009	0.014	0.014	0.013	0.008	0.010	0.007	0.005	0.005	0.001	0.002	0.72 %
		Betaproteobacteria			0.023	0.031	0.035	0.042	0.064	0.058	0.059	0.077	0.055	0.022	0.025	0.018	0.020	4.05 %
			Burkholderiales		0.006	0.015	0.014	0.017	0.036	0.030	0.035	0.052	0.031	0.007	0.013	0.007	0.008	2.09 %
				Comamonadaceae	0.005	0.011	0.013	0.016	0.033	0.028	0.031	0.048	0.029	0.007	0.011	0.007	0.007	1.89 %
			Methylophilales		0.008	0.006	0.008	0.010	0.008	0.012	0.015	0.017	0.015	0.008	0.006	0.005	0.006	0.96 %
				Methylophilaceae	0.008	0.006	0.008	0.010	0.008	0.012	0.015	0.017	0.015	0.008	0.006	0.005	0.006	0.96 %
			Rhodocyclales		0.002	0.003	0.004	0.008	0.012	0.009	0.006	0.004	0.004	0.003	0.001	0.001	0.002	0.45 %
				Rhodocyclaceae	0.002	0.003	0.004	0.008	0.012	0.009	0.006	0.004	0.004	0.003	0.001	0.001	0.002	0.45 %
		Epsilonproteobacteria			0.014	0.019	0.014	0.039	0.066	0.026	0.023	0.019	0.021	0.045	0.015	0.019	0.013	2.57 %
			Campylobacterales		0.014	0.019	0.014	0.039	0.066	0.026	0.023	0.019	0.021	0.045	0.015	0.019	0.013	2.57 %
				Campylobacteraceae	0.014	0.014	0.012	0.032	0.056	0.021	0.021	0.019	0.019	0.045	0.014	0.018	0.011	2.27 %
		Deltaproteobacteria			0.016	0.033	0.053	0.032	0.030	0.043	0.005	0.000	0.006	0.006	0.011	0.019	0.018	2.09 %
	Bacteroidetes				0.228	0.212	0.141	0.179	0.177	0.180	0.233	0.184	0.241	0.185	0.238	0.191	0.213	20.01 %
		Flavobacteriia			0.203	0.169	0.123	0.140	0.129	0.134	0.201	0.163	0.219	0.165	0.207	0.170	0.192	17.04 %
			Flavobacteriales		0.203	0.169	0.123	0.139	0.129	0.134	0.201	0.163	0.219	0.165	0.207	0.170	0.192	17.04 %
				Flavobacteriaceae	0.109	0.086	0.057	0.076	0.068	0.071	0.125	0.109	0.117	0.086	0.101	0.083	0.093	9.09 %
				Cryomorphaceae	0.069	0.060	0.042	0.041	0.049	0.047	0.053	0.045	0.070	0.059	0.089	0.068	0.072	5.87 %
		Bacteroidia			0.005	0.014	0.007	0.018	0.028	0.019	0.008	0.008	0.006	0.008	0.008	0.003	0.005	1.05 %
			Bacteroidales		0.005	0.014	0.007	0.018	0.028	0.019	0.008	0.008	0.006	0.008	0.008	0.003	0.005	1.05 %
	Actinobacteria				0.052	0.040	0.045	0.036	0.024	0.025	0.020	0.031	0.032	0.030	0.026	0.039	0.043	3.39 %
		Actinobacteria			0.022	0.010	0.009	0.002	0.006	0.003	0.011	0.017	0.024	0.016	0.015	0.019	0.023	1.36 %
			Actinomycetales		0.022	0.009	0.008	0.002	0.006	0.002	0.011	0.017	0.023	0.015	0.015	0.019	0.023	1.32 %
				Microbacteriaceae	0.019	0.007	0.003	0.001	0.005	0.001	0.011	0.016	0.021	0.012	0.012	0.016	0.019	1.11 %
		Acidimicrobiia			0.025	0.028	0.035	0.033	0.017	0.022	0.010	0.013	0.008	0.014	0.011	0.019	0.020	1.96 %
			Acidimicrobiales		0.025	0.028	0.035	0.033	0.017	0.022	0.010	0.013	0.008	0.014	0.011	0.019	0.020	1.96 %
				OCS155	0.021	0.021	0.020	0.020	0.010	0.012	0.009	0.012	0.008	0.013	0.009	0.015	0.016	1.42 %
	Verrucomicrobia				0.014	0.015	0.019	0.008	0.008	0.011	0.005	0.001	0.012	0.017	0.016	0.012	0.013	1.17 %
	Cyanobacteria				0.019	0.018	0.018	0.012	0.005	0.005	0.002	0.004	0.015	0.016	0.039	0.033	0.032	1.69 %
		c_Synechococcophycideae			0.018	0.018	0.018	0.012	0.004	0.005	0.002	0.004	0.015	0.016	0.039	0.033	0.032	1.66 %
			Synechococcales		0.018	0.018	0.018	0.012	0.004	0.005	0.002	0.004	0.015	0.016	0.039	0.033	0.032	1.66 %
				Synechococcaceae	0.018	0.018	0.018	0.012	0.004	0.005	0.002	0.004	0.015	0.016	0.039	0.033	0.032	1.66 %
	SAR406				0.009	0.017	0.035	0.021	0.008	0.012	0.007	0.005	0.001	0.001	0.002	0.009	0.009	1.05 %
		c_AB16			0.009	0.017	0.035	0.021	0.008	0.012	0.007	0.005	0.001	0.001	0.002	0.009	0.009	1.05 %

Supplementary 5: Alpha diversity (Shannon) and bacterial abundances (only for U35) evolution along the annual cycle. Bacterial abundances per liter are colored in brown and alpha diversity values (Shannon) for each sample are colored in red (U30), green (U33) and blue (U35). (September 2013 and January 2014 samples were not included as we couldn't quantify that sample by microscopy).



Supplementary 6: Bacterial taxonomic abundances for each water mass. The abundances were calculated by the average value for each genus for each water mass along the annual cycle.

Taxonomy	U30	U33	U35
Unassigned;NA;NA;NA;NA;NA	2.229%	2.173%	1.765%
k_Archaea;p_Crenarchaeota;c_Thaumarchaeota;o_Cenarchaeales;f_Cenarchaeaceae;g_	0.080%	0.076%	0.140%
k_Archaea;p_Crenarchaeota;c_Thaumarchaeota;o_Cenarchaeales;f_Cenarchaeaceae;g_Nitrosopumilus	0.147%	0.146%	0.278%
k_Archaea;p_Euryarchaeota;c_Thermoplasmata;o_E2;f_Marine group II;g_	0.613%	0.739%	1.255%
k_Archaea;p_Euryarchaeota;c_Thermoplasmata;o_E2;f_Marine group III;g_	0.029%	0.032%	0.055%
k_Archaea;p_Parvarchaeota;c_Parvarchaea;o_WCHD3-30;f_;g_	0.006%	0.008%	0.000%
k_Archaea;p_Parvarchaeota;c_Parvarchaea;o_YLA114;f_;g_	0.010%	0.013%	0.005%
p_Acidobacteria;c_AT-s54;o_;f_;g_	0.000%	0.002%	0.007%
p_Acidobacteria;c_Holophagae;o_Holophagales;f_Holophagaceae;g_	0.028%	0.036%	0.007%
p_Acidobacteria;c_OS-K;o_;f_;g_	0.020%	0.009%	0.009%
p_Acidobacteria;c_Sva0725;o_Sva0725;f_;g_	0.005%	0.014%	0.021%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_;g_	0.039%	0.041%	0.134%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_C111;g_	0.094%	0.070%	0.061%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_OCS155;g_	0.696%	0.780%	1.375%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_SC3-41;g_	0.005%	0.006%	0.022%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_TK06;g_	0.058%	0.048%	0.104%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_ZA3409c;g_	0.051%	0.054%	0.121%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_koll13;g_	0.025%	0.024%	0.031%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_ntu14;g_	0.000%	0.000%	0.013%
p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_wb1_P06;g_	0.028%	0.027%	0.064%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_;g_	0.172%	0.143%	0.096%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_ACK-M1;g_	0.007%	0.004%	0.017%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;NA	0.018%	0.020%	0.001%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_	0.801%	0.740%	0.257%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Agrococcus	0.184%	0.178%	0.061%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Candidatus Aquiluna	2.447%	2.276%	0.774%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Candidatus Rhodoluna	0.044%	0.028%	0.007%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Clavibacter	0.040%	0.030%	0.005%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Salinibacterium	0.006%	0.005%	0.006%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Micrococcaceae;g_	0.002%	0.002%	0.005%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Mycobacteriaceae;g_Mycobacterium	0.017%	0.029%	0.044%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Nocardiaceae;g_Rhodococcus	0.012%	0.016%	0.014%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Nocardioidaceae;g_	0.005%	0.007%	0.013%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Nocardioidaceae;g_Aeromicrobium	0.008%	0.015%	0.011%
p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Williamsiaceae;g_Williamsia	0.003%	0.001%	0.007%
p_Actinobacteria;c_Actinobacteria;o_Bifidobacteriales;f_Bifidobacteriaceae;g_Bifidobacterium	0.049%	0.047%	0.033%
p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_Collinsella	0.008%	0.008%	0.006%
p_Actinobacteria;c_Nitriliruptoria;o_Euzebyales;f_Euzebyaceae;g_Euzebya	0.001%	0.013%	0.014%
p_Actinobacteria;c_Thermoleophilia;o_Solirubrobacterales;f_;g_	0.000%	0.002%	0.058%
p_Armatimonadetes;c_Armatimonadia;o_Armatimonadales;f_Armatimonadaceae;g_	0.000%	0.000%	0.004%
p_BHI80-139;c_;o_;f_;g_	0.004%	0.003%	0.004%
p_Bacteroidetes;NA;NA;NA;NA	0.003%	0.004%	0.011%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;NA;NA	0.020%	0.013%	0.029%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_;g_	0.567%	0.453%	0.273%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides	0.561%	0.557%	0.350%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_GZKB119;g_	0.061%	0.039%	0.005%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Marinilabiaceae;g_	0.021%	0.018%	0.016%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_	0.221%	0.207%	0.086%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Paludibacter	0.291%	0.279%	0.095%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Porphyromonadaceae;g_Parabacteroides	0.038%	0.046%	0.019%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella	0.146%	0.150%	0.093%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;NA	0.006%	0.007%	0.003%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_	0.038%	0.034%	0.031%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_AF12	0.000%	0.000%	0.009%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Rikenellaceae;g_Blvii28	0.016%	0.012%	0.008%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g_	0.005%	0.008%	0.000%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_SB-1;g_	0.194%	0.157%	0.083%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_VC21_Bac22;g_	0.031%	0.026%	0.009%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Barnesiellaceae;g_	0.016%	0.016%	0.006%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Odoribacteraceae;g_Butyricimonas	0.008%	0.009%	0.011%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Odoribacteraceae;g_Odoribacter	0.005%	0.005%	0.001%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Paraprevotellaceae;g_	0.005%	0.004%	0.001%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Paraprevotellaceae;g_Paraprevotella	0.007%	0.005%	0.003%
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Paraprevotellaceae;g_Prevotella	0.004%	0.003%	0.006%

Taxonomy	U30	U33	U35
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Cyclobacteriaceae;g_	0.111%	0.072%	0.030%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Cytophagaceae;g_	0.041%	0.038%	0.064%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Cytophagaceae;g_Cytophaga	0.004%	0.005%	0.000%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Cytophagaceae;g_Emticicia	0.006%	0.000%	0.005%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Cytophagaceae;g_Flectobacillus	0.016%	0.014%	0.008%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Cytophagaceae;g_Leadbetterella	0.007%	0.007%	0.004%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Flammeovirgaceae;NA	0.013%	0.023%	0.041%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Flammeovirgaceae;g_	0.185%	0.170%	0.263%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Flammeovirgaceae;g_Flammeovirga	0.003%	0.004%	0.004%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Flammeovirgaceae;g_Flexibacter	0.007%	0.003%	0.000%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Flammeovirgaceae;g_JTB248	0.013%	0.045%	0.118%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Flammeovirgaceae;g_Roseivirga	0.031%	0.033%	0.036%
p_Bacteroidetes;c_Cytophagia;o_Cytophagales;f_Amoebophilaceae;g_Ucs1325	0.004%	0.004%	0.004%
p_Bacteroidetes;c_Flavobacteriia;o_;f_;g_	0.001%	0.001%	0.007%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;NA;NA	0.019%	0.014%	0.066%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_;g_	1.001%	0.961%	1.214%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;NA	0.019%	0.027%	0.033%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;g_	5.332%	5.620%	4.876%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;g_Crocinitomix	0.311%	0.256%	0.135%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;g_Fluviicola	1.059%	0.949%	0.922%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;NA	0.242%	0.263%	0.135%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_	2.588%	2.904%	3.816%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Algibacter	0.007%	0.004%	0.003%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Aquimarina	0.001%	0.000%	0.008%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium	3.239%	3.242%	2.896%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Gaetbulibacter	0.007%	0.012%	0.008%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Gelidibacter	0.010%	0.013%	0.005%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Gramella	0.005%	0.007%	0.003%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Kordia	0.006%	0.006%	0.011%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Maribacter	0.017%	0.010%	0.006%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Mesonia	0.001%	0.006%	0.007%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Polaribacter	1.059%	1.193%	1.016%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Robiginitalea	0.011%	0.015%	0.006%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Salegentibacter	0.007%	0.002%	0.016%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Sediminicola	1.330%	1.306%	0.689%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Tenacibaculum	0.238%	0.218%	0.334%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Winogradskyella	0.026%	0.024%	0.051%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_NS9;g_	0.515%	0.547%	0.725%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Weeksellaceae;g_Chryseobacterium	0.007%	0.005%	0.005%
p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Weeksellaceae;g_Cloacibacterium	0.083%	0.060%	0.031%
p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_;g_	0.376%	0.341%	0.186%
p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_NS11-12;g_	0.462%	0.485%	0.353%
p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Sphingobacteriaceae;g_	0.006%	0.008%	0.003%
p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales;f_Sphingobacteriaceae;g_Pedobacter	0.020%	0.019%	0.009%
p_Bacteroidetes;c_Rhodothermi;o_Rhodothermales;f_Rhodothermaceae;g_	0.031%	0.018%	0.026%
p_Bacteroidetes;c_Rhodothermi;o_Rhodothermales;f_Balneolaceae;g_	0.009%	0.004%	0.002%
p_Bacteroidetes;c_Rhodothermi;o_Rhodothermales;f_Balneolaceae;g_Balneola	0.112%	0.100%	0.119%
p_Bacteroidetes;c_Saprospirae;o_Saprospirales;f_;g_	0.043%	0.036%	0.054%
p_Bacteroidetes;c_Saprospirae;o_Saprospirales;f_Chitinophagaceae;g_	0.011%	0.008%	0.012%
p_Bacteroidetes;c_Saprospirae;o_Saprospirales;f_Chitinophagaceae;g_Sediminibacterium	0.041%	0.047%	0.063%
p_Bacteroidetes;c_Saprospirae;o_Saprospirales;f_Saprospiraceae;g_	0.308%	0.294%	0.480%
p_Bacteroidetes;c_Saprospirae;o_Saprospirales;f_Saprospiraceae;g_Lewinella	0.018%	0.027%	0.050%
p_Bacteroidetes;c_Saprospirae;o_Saprospirales;f_Saprospiraceae;g_Saprospira	0.031%	0.029%	0.082%
p_Caldithrix;c_Caldithrixae;o_Caldithrixales;f_Caldithrixaceae;g_	0.007%	0.001%	0.002%
p_Caldithrix;c_Caldithrixae;o_Caldithrixales;f_Caldithrixaceae;g_LCP-26	0.008%	0.015%	0.006%
p_Chlamydiae;c_Chlamydiia;o_Chlamydiales;f_;g_	0.004%	0.007%	0.001%
p_Chlorobi;c_Ignavibacteria;o_Ignavibacteriales;f_Ignavibacteriaceae;g_	0.009%	0.007%	0.008%
p_Chlorobi;c_Ignavibacteria;o_Ignavibacteriales;f_IheB3-7;g_	0.004%	0.003%	0.003%
p_Chlorobi;c_OPB56;o_;f_;g_	0.017%	0.018%	0.005%
p_Chloroflexi;c_Anaerolineae;o_GCA004;f_;g_	0.023%	0.024%	0.013%
p_Chloroflexi;c_Anaerolineae;o_SBR1031;f_A4b;g_	0.002%	0.006%	0.007%
p_Chloroflexi;c_Anaerolineae;o_SHA-20;f_;g_	0.002%	0.004%	0.004%
p_Chloroflexi;c_SAR202;o_;f_;g_	0.128%	0.101%	0.243%
p_Chloroflexi;c_TK17;o_;f_;g_	0.013%	0.015%	0.025%
p_Cyanobacteria;c_4C0d-2;o_MLE1-12;f_;g_	0.000%	0.001%	0.010%
p_Cyanobacteria;c_4C0d-2;o_SM1D11;f_;g_	0.000%	0.001%	0.008%
p_Cyanobacteria;c_4C0d-2;o_YS2;f_;g_	0.039%	0.030%	0.015%
p_Cyanobacteria;c_Oscillatoriophycideae;o_Chroococcales;f_Xenococcaceae;g_	0.003%	0.010%	0.009%
p_Cyanobacteria;c_Synechococcophycideae;o_Synechococcales;f_Synechococcaceae;g_Prochlorococcus	0.075%	0.087%	0.250%
p_Cyanobacteria;c_Synechococcophycideae;o_Synechococcales;f_Synechococcaceae;g_Synechococcus	0.286%	0.488%	1.376%
p_Elusimicrobia;c_Elusimicrobia;o_Elusimicrobiales;f_Elusimicrobiaceae;g_	0.013%	0.008%	0.000%

Taxonomy	U30	U33	U35
p_Fibrobacteres;c_;o_;f_;g_	0.027%	0.030%	0.005%
p_Fibrobacteres;c_Fibrobacteria;o_258ds10;f_;g_	0.003%	0.005%	0.001%
p_Fibrobacteres;c_Fibrobacteria;o_Fibrobacterales;f_;g_	0.013%	0.009%	0.017%
p_Fibrobacteres;c_Fibrobacteria;o_Fibrobacterales;f_Fibrobacteraceae;g_Fibrobacter	0.017%	0.013%	0.002%
p_Fibrobacteres;c_TG3;o_TG3-2;f_;g_	0.006%	0.007%	0.001%
p_Firmicutes;c_Bacilli;o_Bacillales;f_Staphylococcaceae;g_Staphylococcus	0.006%	0.006%	0.012%
p_Firmicutes;c_Bacilli;o_Lactobacillales;NA;NA	0.005%	0.007%	0.005%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Carnobacteriaceae;g_	0.024%	0.033%	0.026%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Lactococcus	0.002%	0.004%	0.006%
p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus	0.007%	0.008%	0.014%
p_Firmicutes;c_Clostridia;o_Clostridiales;NA;NA	0.005%	0.005%	0.000%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_;g_	0.157%	0.117%	0.086%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g_	0.006%	0.006%	0.000%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_	0.024%	0.036%	0.013%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Clostridiaceae;g_Clostridium	0.064%	0.080%	0.024%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Eubacteriaceae;g_Acetobacterium	0.012%	0.010%	0.003%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_	0.087%	0.106%	0.054%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Blautia	0.036%	0.047%	0.027%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Coprococcus	0.028%	0.025%	0.023%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Dorea	0.003%	0.004%	0.001%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Lachnospira	0.015%	0.012%	0.001%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Lachnospiraceae;g_Ruminococcus	0.020%	0.017%	0.014%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Peptostreptococcaceae;g_	0.069%	0.053%	0.027%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_	0.084%	0.087%	0.043%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium	0.064%	0.073%	0.068%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Ruminococcaceae;g_Ruminococcus	0.033%	0.037%	0.020%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;NA	0.004%	0.006%	0.001%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_	0.053%	0.036%	0.024%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Anaeromusa	0.004%	0.013%	0.002%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Dialister	0.022%	0.022%	0.014%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Megamonas	0.010%	0.012%	0.003%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Pelosinus	0.005%	0.010%	0.003%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Phascolarctobacterium	0.014%	0.011%	0.004%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_vadinHB04	0.020%	0.020%	0.004%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Acidaminobacteraceae;g_Fusibacter	0.020%	0.009%	0.009%
p_Firmicutes;c_Clostridia;o_Clostridiales;f_Acidaminobacteraceae;g_WH1-8	0.006%	0.013%	0.010%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_	0.010%	0.012%	0.008%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Catenibacterium	0.004%	0.011%	0.006%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_PSB-M-3	0.007%	0.003%	0.000%
p_Firmicutes;c_Erysipelotrichi;o_Erysipelotrichales;f_Erysipelotrichaceae;g_Eubacterium	0.013%	0.017%	0.015%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_;g_	0.162%	0.159%	0.081%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Fusobacteriaceae;g_Cetobacterium	0.004%	0.004%	0.019%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Fusobacteriaceae;g_Fusobacterium	0.012%	0.010%	0.022%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Fusobacteriaceae;g_Propionigenium	0.038%	0.041%	0.060%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Fusobacteriaceae;g_Psychrilyobacter	0.008%	0.008%	0.018%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Fusobacteriaceae;g_u114	0.047%	0.060%	0.018%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Leptotrichiaceae;NA	0.004%	0.004%	0.001%
p_Fusobacteria;c_Fusobacteriia;o_Fusobacteriales;f_Leptotrichiaceae;g_	0.025%	0.016%	0.012%
p_GN02;c_;o_;f_;g_	0.009%	0.005%	0.002%
p_GN02;c_BD1-5;o_;f_;g_	0.073%	0.060%	0.034%
p_Gemmatimonadetes;c_Gemm-2;o_;f_;g_	0.107%	0.103%	0.165%
p_Gemmatimonadetes;c_Gemm-4;o_;f_;g_	0.032%	0.029%	0.020%
p_H-178;c_;o_;f_;g_	0.006%	0.003%	0.001%
p_Lentisphaerae;c_Lentisphaeria;o_Lentisphaerales;f_Lentisphaeraceae;g_	0.003%	0.001%	0.008%
p_Lentisphaerae;c_Lentisphaeria;o_Lentisphaerales;f_Lentisphaeraceae;g_Lentisphaera	0.014%	0.007%	0.006%
p_Lentisphaerae;c_Lentisphaeria;o_Victivallales;f_Victivallaceae;g_	0.019%	0.017%	0.007%
p_Nitrospirae;c_Nitrospira;o_Nitrospirales;f_Nitrospiraceae;g_	0.000%	0.005%	0.009%
p_Nitrospirae;c_Nitrospira;o_Nitrospirales;f_Nitrospiraceae;g_Nitrospira	0.004%	0.003%	0.001%
p_Nitrospirae;c_Nitrospira;o_Nitrospirales;f_Thermodesulfovibrionaceae;g_BD2-6	0.002%	0.000%	0.006%
p_OD1;c_ZB2;o_;f_;g_	0.015%	0.014%	0.006%
p_OP3;c_PBS-25;o_;f_;g_	0.043%	0.032%	0.007%
p_OP8;c_;o_;f_;g_	0.008%	0.011%	0.000%
p_PAUC34f;c_;o_;f_;g_	0.017%	0.013%	0.039%
p_Planctomycetes;c_028H05-P-BN-P5;o_;f_;g_	0.001%	0.000%	0.007%
p_Planctomycetes;c_OM190;o_CL500-15;f_;g_	0.017%	0.015%	0.060%
p_Planctomycetes;c_OM190;o_agg27;f_;g_	0.001%	0.004%	0.021%
p_Planctomycetes;c_Phycisphaerae;o_MSBL9;f_;g_	0.013%	0.014%	0.006%
p_Planctomycetes;c_Phycisphaerae;o_Phycisphaerales;f_;g_	0.015%	0.011%	0.095%
p_Planctomycetes;c_Pla3;o_;f_;g_	0.047%	0.059%	0.104%
p_Planctomycetes;c_Planctomycetia;o_Pirellulales;f_Pirellulaceae;g_	0.085%	0.087%	0.276%
p_Planctomycetes;c_Planctomycetia;o_Planctomycetales;f_Planctomycetaceae;g_Planctomyces	0.024%	0.031%	0.051%

Taxonomy	U30	U33	U35
p_Planctomycetes;c_vadinHA49;o_;f_;g_	0.004%	0.008%	0.004%
p_Proteobacteria;NA;NA;NA;NA	0.020%	0.019%	0.012%
p_Proteobacteria;c_Alphaproteobacteria;NA;NA;NA	0.038%	0.028%	0.107%
p_Proteobacteria;c_Alphaproteobacteria;o_;f_;g_	4.341%	4.635%	5.958%
p_Proteobacteria;c_Alphaproteobacteria;o_BD7-3;f_;g_	0.006%	0.020%	0.032%
p_Proteobacteria;c_Alphaproteobacteria;o_Caulobacterales;f_Caulobacteraceae;g_	0.011%	0.032%	0.054%
p_Proteobacteria;c_Alphaproteobacteria;o_Caulobacterales;f_Caulobacteraceae;g_Caulobacter	0.004%	0.014%	0.017%
p_Proteobacteria;c_Alphaproteobacteria;o_Caulobacterales;f_Caulobacteraceae;g_Mycoplana	0.029%	0.027%	0.060%
p_Proteobacteria;c_Alphaproteobacteria;o_Caulobacterales;f_Caulobacteraceae;g_Phenylobacterium	0.016%	0.021%	0.046%
p_Proteobacteria;c_Alphaproteobacteria;o_Ellin329;f_;g_	0.000%	0.003%	0.006%
p_Proteobacteria;c_Alphaproteobacteria;o_Kiloniellales;f_;g_	0.061%	0.089%	0.186%
p_Proteobacteria;c_Alphaproteobacteria;o_Kiloniellales;f_Kiloniellaceae;g_	0.168%	0.163%	0.080%
p_Proteobacteria;c_Alphaproteobacteria;o_Kiloniellales;f_Kiloniellaceae;g_Thalassospira	0.018%	0.016%	0.020%
p_Proteobacteria;c_Alphaproteobacteria;o_Kordiimonadales;f_Kordiimonadaceae;g_	0.001%	0.004%	0.004%
p_Proteobacteria;c_Alphaproteobacteria;o_RF32;f_;g_	0.037%	0.038%	0.020%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_;g_	0.172%	0.142%	0.147%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Aurantimonadaceae;g_	0.009%	0.007%	0.038%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Bradyrhizobiaceae;g_	0.008%	0.005%	0.014%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Hyphomicrobiaceae;g_	0.025%	0.030%	0.055%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Hyphomicrobiaceae;g_Devosia	0.019%	0.016%	0.028%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Hyphomicrobiaceae;g_Rhodoplanes	0.019%	0.011%	0.009%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Phyllobacteriaceae;NA	0.011%	0.009%	0.010%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Phyllobacteriaceae;g_	0.047%	0.030%	0.136%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Phyllobacteriaceae;g_Aminobacter	0.000%	0.004%	0.005%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhizobiales;f_Rhizobiaceae;g_Agrobacterium	0.019%	0.020%	0.018%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Hyphomonadaceae;g_	0.237%	0.261%	0.410%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Hyphomonadaceae;g_Hyphomonas	0.020%	0.028%	0.067%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Hyphomonadaceae;g_Maricaulis	0.006%	0.007%	0.026%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Hyphomonadaceae;g_Oceanicaulis	0.010%	0.008%	0.017%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;NA	2.747%	3.125%	2.199%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_	10.197%	10.657%	9.634%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Anaerospora	0.353%	0.354%	0.277%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Loktanella	0.191%	0.203%	0.417%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Marivita	0.093%	0.063%	0.085%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Oceanicola	0.005%	0.006%	0.005%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter	2.164%	2.316%	1.733%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Paracoccus	0.023%	0.018%	0.010%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Phaeobacter	0.010%	0.000%	0.008%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria	2.118%	2.066%	2.158%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Rhodobacter	1.190%	1.106%	0.583%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Roseivivax	0.202%	0.206%	0.139%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Roseobacter	0.012%	0.012%	0.002%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_;g_	0.004%	0.008%	0.001%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_Rhodospirillaceae;g_	0.422%	0.409%	0.829%
p_Proteobacteria;c_Alphaproteobacteria;o_Rhodospirillales;f_Rhodospirillaceae;g_Nisaea	0.004%	0.004%	0.010%
p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_;g_	0.412%	0.423%	0.569%
p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_AEGEAN_112;g_	0.103%	0.137%	0.286%
p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Pelagibacteraceae;g_	1.751%	1.934%	3.229%
p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Rickettsiaceae;g_	0.122%	0.082%	0.051%
p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_mitochondria;g_	0.059%	0.078%	0.185%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_;g_	0.182%	0.190%	0.243%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Erythrobacteraceae;NA	0.102%	0.088%	0.048%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Erythrobacteraceae;g_	0.232%	0.229%	0.126%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Erythrobacteraceae;g_Erythrobacter	0.018%	0.019%	0.018%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;NA	0.012%	0.024%	0.043%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_	0.016%	0.021%	0.039%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_Kaistobacter	0.009%	0.011%	0.003%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_Novosphingobium	0.087%	0.074%	0.029%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_Sphingobium	0.024%	0.022%	0.020%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_Sphingomonas	0.015%	0.028%	0.033%
p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales;f_Sphingomonadaceae;g_Sphingopyxis	0.017%	0.006%	0.032%
p_Proteobacteria;c_Betaproteobacteria;NA;NA;NA	0.222%	0.206%	0.101%
p_Proteobacteria;c_Betaproteobacteria;o_;f_;g_	0.206%	0.187%	0.183%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;NA;NA	0.004%	0.003%	0.003%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_;g_	0.011%	0.013%	0.009%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_	0.127%	0.096%	0.023%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Denitrobacter	0.004%	0.005%	0.000%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Alcaligenaceae;g_Sutterella	0.029%	0.032%	0.011%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Burkholderiaceae;g_Burkholderia	0.007%	0.008%	0.001%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;NA	1.135%	0.852%	0.336%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_	1.491%	1.228%	0.695%

Taxonomy	U30	U33	U35
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Acidovorax	0.005%	0.006%	0.024%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Comamonas	0.047%	0.032%	0.024%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Hydrogenophaga	0.211%	0.185%	0.083%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Limnobacter	0.094%	0.125%	0.120%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Limnohabitans	0.087%	0.083%	0.075%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Methylibium	0.013%	0.014%	0.007%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_RS62	1.661%	1.523%	0.578%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Rhodoferax	0.005%	0.001%	0.008%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_Simplicispira	0.016%	0.014%	0.009%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae;NA	0.028%	0.022%	0.010%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae;g_	0.059%	0.055%	0.045%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae;g_Janthinobacterium	0.028%	0.034%	0.020%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae;g_Polynucleobacter	0.068%	0.048%	0.050%
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Oxalobacteraceae;g_Ralstonia	0.012%	0.021%	0.029%
p_Proteobacteria;c_Betaproteobacteria;o_Gallionellales;f_Gallionellaceae;g_Gallionella	0.003%	0.006%	0.000%
p_Proteobacteria;c_Betaproteobacteria;o_MND1;f_;g_	0.004%	0.003%	0.003%
p_Proteobacteria;c_Betaproteobacteria;o_MWH-UniP1;f_;g_	0.133%	0.102%	0.117%
p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;NA;NA	0.007%	0.010%	0.000%
p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;f_Methylophilaceae;NA	0.055%	0.049%	0.005%
p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;f_Methylophilaceae;g_	1.052%	0.996%	0.727%
p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;f_Methylophilaceae;g_Methylotenera	0.405%	0.355%	0.193%
p_Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;NA	0.020%	0.015%	0.006%
p_Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;g_	0.030%	0.019%	0.007%
p_Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;g_Microvirgula	0.034%	0.032%	0.020%
p_Proteobacteria;c_Betaproteobacteria;o_Neisseriales;f_Neisseriaceae;g_Vogesella	0.005%	0.004%	0.000%
p_Proteobacteria;c_Betaproteobacteria;o_Nitrosomonadales;f_Nitrosomonadaceae;g_	0.012%	0.011%	0.012%
p_Proteobacteria;c_Betaproteobacteria;o_Procabacteriales;f_Procabacteriaceae;g_	0.184%	0.199%	0.093%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;NA	0.054%	0.053%	0.016%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_	0.655%	0.566%	0.255%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_C39	0.016%	0.015%	0.017%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Candidatus Accumulibacter	0.018%	0.019%	0.006%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Dechloromonas	0.253%	0.211%	0.063%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Propionivibrio	0.184%	0.172%	0.092%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Thauera	0.029%	0.027%	0.005%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Uliginosibacterium	0.028%	0.022%	0.007%
p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Zoogloea	0.061%	0.066%	0.024%
p_Proteobacteria;c_Betaproteobacteria;o_SBla14;f_;g_	0.026%	0.018%	0.011%
p_Proteobacteria;c_Deltaproteobacteria;o_;f_;g_	0.053%	0.052%	0.097%
p_Proteobacteria;c_Deltaproteobacteria;o_Bdellovibrionales;f_Bacteriovoracaceae;g_	0.173%	0.173%	0.130%
p_Proteobacteria;c_Deltaproteobacteria;o_Bdellovibrionales;f_Bacteriovoracaceae;g_Bacteriovorax	0.117%	0.113%	0.074%
p_Proteobacteria;c_Deltaproteobacteria;o_Bdellovibrionales;f_Bdellovibrionaceae;g_Bdellovibrio	0.004%	0.009%	0.008%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfarculales;f_Desulfarculaceae;g_	0.027%	0.030%	0.030%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Desulfobacteraceae;g_	0.121%	0.104%	0.087%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Desulfobacteraceae;g_Desulfococcus	0.112%	0.136%	0.073%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Desulfobacteraceae;g_Desulfosarcina	0.036%	0.037%	0.009%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Desulfobulbaceae;g_	0.441%	0.380%	0.220%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Desulfobulbaceae;g_Desulfobulbus	0.030%	0.025%	0.008%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Desulfobulbaceae;g_Desulfocapsa	0.004%	0.003%	0.003%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Desulfobulbaceae;g_Desulfotalea	0.003%	0.009%	0.004%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfobacterales;f_Nitrospinaceae;g_Nitrospina	0.037%	0.030%	0.073%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfomicrobiaceae;g_Desulfomicrobium	0.013%	0.014%	0.012%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfovibrionaceae;g_	0.012%	0.026%	0.020%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfovibrionaceae;g_Bilophila	0.018%	0.016%	0.008%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales;f_Desulfovibrionaceae;g_Desulfovibrio	0.067%	0.061%	0.042%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfuromonadales;f_Desulfuromonadaceae;g_	0.197%	0.194%	0.076%
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfuromonadales;f_Geobacteraceae;g_Geobacter	0.017%	0.011%	0.002%
p_Proteobacteria;c_Deltaproteobacteria;o_GMD14H09;f_;g_	0.023%	0.026%	0.035%
p_Proteobacteria;c_Deltaproteobacteria;o_GW-28;f_;g_	0.005%	0.005%	0.002%
p_Proteobacteria;c_Deltaproteobacteria;o_MBNT15;f_;g_	0.005%	0.008%	0.000%
p_Proteobacteria;c_Deltaproteobacteria;o_Myxococcales;f_;g_	0.069%	0.071%	0.147%
p_Proteobacteria;c_Deltaproteobacteria;o_Myxococcales;f_Cystobacterineae;g_	0.008%	0.006%	0.024%
p_Proteobacteria;c_Deltaproteobacteria;o_Myxococcales;f_Haliangiaceae;g_	0.006%	0.010%	0.012%
p_Proteobacteria;c_Deltaproteobacteria;o_Myxococcales;f_Nannocystaceae;g_Plesiocystis	0.012%	0.009%	0.027%
p_Proteobacteria;c_Deltaproteobacteria;o_Myxococcales;f_OM27;g_	0.304%	0.270%	0.340%
p_Proteobacteria;c_Deltaproteobacteria;o_NB1-j;f_JTB38;g_	0.029%	0.042%	0.080%
p_Proteobacteria;c_Deltaproteobacteria;o_PB19;f_;g_	0.397%	0.327%	0.170%
p_Proteobacteria;c_Deltaproteobacteria;o_Spirobacillales;f_;g_	0.037%	0.032%	0.094%
p_Proteobacteria;c_Deltaproteobacteria;o_Sva0853;f_;g_	0.008%	0.005%	0.027%
p_Proteobacteria;c_Deltaproteobacteria;o_Sva0853;f_S25_1238;g_	0.067%	0.070%	0.129%
p_Proteobacteria;c_Deltaproteobacteria;o_Sva0853;f_SAR324;g_	0.042%	0.036%	0.074%
p_Proteobacteria;c_Deltaproteobacteria;o_Syntrophobacterales;f_Syntrophobacteraceae;g_	0.026%	0.030%	0.062%

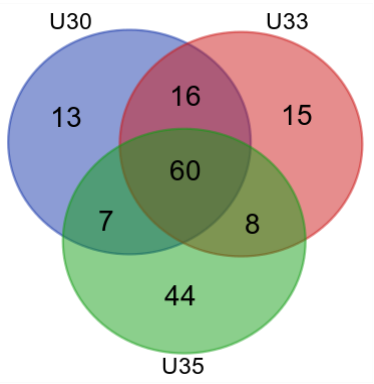
Taxonomy	U30	U33	U35
p_Proteobacteria;c_Deltaproteobacteria;o_Entotheonellales;f_;g_	0.004%	0.003%	0.007%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_;g_	0.007%	0.014%	0.006%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;NA	0.003%	0.006%	0.000%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_	0.016%	0.012%	0.007%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter	2.403%	2.237%	2.163%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Sulfurospirillum	0.205%	0.192%	0.097%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Helicobacteraceae;g_	0.340%	0.336%	0.249%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Helicobacteraceae;g_Sulfuricurvum	0.043%	0.057%	0.024%
p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Helicobacteraceae;g_Sulfurimonas	0.183%	0.143%	0.065%
p_Proteobacteria;c_Gammaproteobacteria;NA;NA;NA	0.315%	0.312%	0.272%
p_Proteobacteria;c_Gammaproteobacteria;o_;f_;g_	0.116%	0.138%	0.204%
p_Proteobacteria;c_Gammaproteobacteria;o_34P16;f_;g_	0.005%	0.018%	0.028%
p_Proteobacteria;c_Gammaproteobacteria;o_Acidithiobacillales;f_;g_	0.025%	0.029%	0.046%
p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Aeromonadaceae;NA	0.082%	0.061%	0.009%
p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Aeromonadaceae;g_	0.497%	0.485%	0.286%
p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Aeromonadaceae;g_Tolumonas	0.224%	0.210%	0.115%
p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Succinivibrionaceae;g_Succinivibrio	0.050%	0.045%	0.021%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;NA;NA	0.120%	0.134%	0.152%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_;g_	0.085%	0.092%	0.283%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;NA	0.304%	0.247%	0.200%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_	0.403%	0.389%	0.282%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Agarivorans	0.006%	0.000%	0.022%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Alteromonas	0.067%	0.072%	0.091%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_BD2-13	0.112%	0.097%	0.062%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Candidatus Endobugula	0.035%	0.038%	0.037%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Cellvibrio	0.020%	0.018%	0.009%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Glaciecola	1.680%	1.996%	2.346%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_HB2-32-21	0.000%	0.008%	0.043%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_HTCC2207	1.218%	1.127%	0.945%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Marinobacter	0.067%	0.114%	0.157%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Microbulbifer	0.002%	0.003%	0.006%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_ZD0117	0.071%	0.063%	0.086%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_nsmpVI18	0.056%	0.052%	0.011%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Colwelliaceae;g_	0.199%	0.202%	0.416%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Colwelliaceae;g_Thalassomonas	0.132%	0.148%	0.305%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Ferrimonadaceae;g_Ferrimonas	0.008%	0.002%	0.025%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_	0.053%	0.054%	0.094%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC	2.874%	2.526%	1.681%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Idiomarinaceae;g_	0.001%	0.000%	0.012%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Idiomarinaceae;g_Idiomarina	0.043%	0.085%	0.077%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Idiomarinaceae;g_Pseudidiomarina	0.018%	0.013%	0.006%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_J115;NA	0.020%	0.032%	0.059%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_J115;g_	0.010%	0.034%	0.016%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Moritellaceae;g_Moritella	0.004%	0.009%	0.013%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60;g_	2.782%	2.904%	2.786%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60;g_Congregibacter	0.086%	0.078%	0.068%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Psychromonadaceae;g_Psychromonas	0.050%	0.061%	0.083%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Shewanellaceae;g_Shewanella	0.160%	0.140%	0.191%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Chromatiaceae;NA	0.025%	0.018%	0.010%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Chromatiaceae;g_	0.004%	0.006%	0.002%
p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Chromatiaceae;g_Rheinheimera	0.090%	0.085%	0.043%
p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales;f_;g_	1.311%	1.160%	0.734%
p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales;f_Chromatiaceae;g_	0.061%	0.046%	0.028%
p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales;f_Ectothiorhodospiraceae;g_	0.021%	0.021%	0.082%
p_Proteobacteria;c_Gammaproteobacteria;o_Chromatiales;f_Halothiobacillaceae;g_Thiovirga	0.006%	0.004%	0.001%
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;NA	0.078%	0.060%	0.025%
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_	0.179%	0.163%	0.118%
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Citrobacter	0.034%	0.035%	0.029%
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Erwinia	0.034%	0.029%	0.007%
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Plesiomonas	0.013%	0.011%	0.003%
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales;f_Enterobacteriaceae;g_Serratia	0.018%	0.011%	0.003%
p_Proteobacteria;c_Gammaproteobacteria;o_HOC36;f_;g_	0.012%	0.016%	0.009%
p_Proteobacteria;c_Gammaproteobacteria;o_HTCC2188;f_;g_	0.337%	0.320%	0.138%
p_Proteobacteria;c_Gammaproteobacteria;o_HTCC2188;f_HTCC2089;g_	0.181%	0.256%	0.490%
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_;g_	0.028%	0.029%	0.054%
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_Coxiellaceae;g_	0.058%	0.075%	0.138%
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_Coxiellaceae;g_Rickettsiella	0.059%	0.065%	0.032%
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_Endoecteinascidiaceae;g_	0.001%	0.004%	0.009%
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_Francisellaceae;g_	0.076%	0.055%	0.031%
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_Francisellaceae;g_Francisella	0.028%	0.038%	0.011%
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_Legionellaceae;g_	0.000%	0.001%	0.005%

Taxonomy	U30	U33	U35
p_Proteobacteria;c_Gammaproteobacteria;o_Legionellales;f_Legionellaceae;g_Tatlockia	0.000%	0.000%	0.009%
p_Proteobacteria;c_Gammaproteobacteria;o_Methylococcales;f_;g_	0.005%	0.006%	0.002%
p_Proteobacteria;c_Gammaproteobacteria;o_Methylococcales;f_Methylococcaceae;g_Methylomicrobium	0.002%	0.001%	0.006%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;NA;NA	0.054%	0.035%	0.040%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_;g_	1.045%	1.107%	0.805%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Alcanivoracaceae;g_Alcanivorax	0.109%	0.160%	0.242%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Endozoicimonaceae;g_	0.065%	0.040%	0.102%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae;g_	0.003%	0.003%	0.006%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae;g_Candidatus Portiera	1.578%	1.707%	3.164%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae;g_Halomonas	0.061%	0.049%	0.094%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;NA	0.076%	0.066%	0.307%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_	1.416%	1.487%	2.314%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Amphritea	0.013%	0.017%	0.016%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Marinobacterium	0.007%	0.008%	0.006%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Marinomonas	0.267%	0.274%	0.223%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Neptunomonas	0.047%	0.034%	0.034%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Oceanospirillum	0.009%	0.004%	0.009%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Oleibacter	0.046%	0.040%	0.036%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Oleispira	0.193%	0.175%	0.618%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oleiphilaceae;g_	0.210%	0.186%	0.471%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_SUP05;g_	0.059%	0.037%	0.089%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Saccharospirillaceae;NA	0.008%	0.011%	0.008%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Saccharospirillaceae;g_	0.062%	0.057%	0.021%
p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Saccharospirillaceae;g_Reinekea	0.027%	0.019%	0.011%
p_Proteobacteria;c_Gammaproteobacteria;o_PYR10d3;f_;g_	0.006%	0.007%	0.007%
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_	0.057%	0.055%	0.019%
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter	0.705%	0.581%	0.380%
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Perlucidibaca	0.030%	0.013%	0.002%
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Psychrobacter	0.010%	0.024%	0.036%
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_	0.064%	0.070%	0.072%
p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Pseudomonadaceae;g_Pseudomonas	0.389%	0.356%	0.238%
p_Proteobacteria;c_Gammaproteobacteria;o_Salinisphaerales;f_;g_	0.086%	0.088%	0.157%
p_Proteobacteria;c_Gammaproteobacteria;o_Salinisphaerales;f_Salinisphaeraceae;NA	0.000%	0.001%	0.009%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiohalorhabdales;f_;g_	0.108%	0.129%	0.221%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiohalorhabdales;f_Thiohalorhabdaceae;g_	0.095%	0.104%	0.203%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_;g_	0.017%	0.011%	0.012%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_Piscirickettsiaceae;NA	0.009%	0.012%	0.024%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_Piscirickettsiaceae;g_	0.700%	0.712%	1.112%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_Thiotrichaceae;g_	0.130%	0.112%	0.117%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_Thiotrichaceae;g_B46	0.002%	0.006%	0.014%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_Thiotrichaceae;g_Leucothrix	0.007%	0.006%	0.030%
p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_Thiotrichaceae;g_Thiothrix	0.014%	0.018%	0.045%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Pseudoalteromonadaceae;g_	0.060%	0.053%	0.086%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Pseudoalteromonadaceae;g_Pseudoalteromonas	0.211%	0.216%	0.788%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;NA	0.152%	0.151%	0.428%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;g_	0.051%	0.051%	0.070%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;g_Aliivibrio	0.052%	0.041%	0.061%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;g_Enterovibrio	0.019%	0.022%	0.039%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;g_Photobacterium	0.057%	0.030%	0.100%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;g_Salinivibrio	0.007%	0.013%	0.000%
p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae;g_Vibrio	0.148%	0.145%	0.296%
p_Proteobacteria;c_Gammaproteobacteria;o_Xanthomonadales;f_Sinobacteraceae;g_	0.039%	0.093%	0.155%
p_Proteobacteria;c_Gammaproteobacteria;o_Xanthomonadales;f_Sinobacteraceae;g_Hydrocarboniphaga	0.011%	0.000%	0.007%
p_Proteobacteria;c_Gammaproteobacteria;o_Xanthomonadales;f_Sinobacteraceae;g_Nevskia	0.002%	0.000%	0.010%
p_Proteobacteria;c_Gammaproteobacteria;o_Xanthomonadales;f_Xanthomonadaceae;NA	0.002%	0.003%	0.003%
p_Proteobacteria;c_Gammaproteobacteria;o_Xanthomonadales;f_Xanthomonadaceae;g_	0.018%	0.017%	0.004%
p_Proteobacteria;c_Gammaproteobacteria;o_Marinicellales;f_Marinicellaceae;g_	0.309%	0.278%	0.250%
p_Proteobacteria;c_Gammaproteobacteria;o_Marinicellales;f_Marinicellaceae;g_Marinicella	0.024%	0.013%	0.027%
p_SAR406;c_AB16;o_Arctic96B-7;f_A714017;g_Arctic95A-2	0.004%	0.008%	0.011%
p_SAR406;c_AB16;o_Arctic96B-7;f_A714017;g_SGSH944	0.127%	0.120%	0.411%
p_SAR406;c_AB16;o_Arctic96B-7;f_A714017;g_SargSea-WGS	0.068%	0.061%	0.112%
p_SAR406;c_AB16;o_Arctic96B-7;f_A714017;g_ZA3312c	0.193%	0.212%	0.372%
p_SAR406;c_AB16;o_Arctic96B-7;f_A714017;g_so4B24	0.001%	0.004%	0.007%
p_SAR406;c_AB16;o_Arctic96B-7;f_Sc-NB04;g_	0.012%	0.008%	0.017%
p_SAR406;c_AB16;o_ZA3648c;f_AEGEAN_185;g_	0.028%	0.030%	0.081%
p_SBR1093;c_A712011;o_;f_;g_	0.074%	0.076%	0.129%
p_Spirochaetes;c_MVP-15;o_PL-11B10;f_;g_	0.012%	0.016%	0.000%
p_Spirochaetes;c_Spirochaetes;o_Spirochaetales;f_Spirochaetaceae;g_Spirochaeta	0.019%	0.014%	0.006%
p_Spirochaetes;c_Spirochaetes;o_Spirochaetales;f_Spirochaetaceae;g_Treponema	0.025%	0.027%	0.005%
p_Synergistetes;c_Synergistia;o_Synergistales;f_Dethiosulfovibrionaceae;g_PD-UASB-13	0.006%	0.005%	0.002%
p_Synergistetes;c_Synergistia;o_Synergistales;f_Synergistaceae;g_vadinCA02	0.055%	0.053%	0.018%

Taxonomy	U30	U33	U35
p_Tenericutes;c_CK-1C4-19;o_;f_;g_	0.000%	0.000%	0.012%
p_Tenericutes;c_Mollicutes;o_;f_;g_	0.040%	0.028%	0.008%
p_Tenericutes;c_Mollicutes;o_Acholeplasmatales;f_Acholeplasmataceae;g_Acholeplasma	0.146%	0.118%	0.021%
p_Tenericutes;c_Mollicutes;o_Mycoplasmatales;f_Mycoplasmataceae;g_Candidatus Hepatoplasma	0.019%	0.013%	0.000%
p_Tenericutes;c_Mollicutes;o_RF39;f_;g_	0.003%	0.007%	0.002%
p_Verrucomicrobia;c_Opitutae;o_;f_;g_	0.025%	0.015%	0.019%
p_Verrucomicrobia;c_Opitutae;o_Opitutales;f_Opitutaceae;g_	0.050%	0.039%	0.021%
p_Verrucomicrobia;c_Opitutae;o_Opitutales;f_Opitutaceae;g_Opitutus	0.052%	0.041%	0.014%
p_Verrucomicrobia;c_Opitutae;o_Puniceicoccales;f_Puniceicoccaceae;g_	0.015%	0.010%	0.008%
p_Verrucomicrobia;c_Opitutae;o_Puniceicoccales;f_Puniceicoccaceae;g_Coraliomargarita	0.125%	0.159%	0.180%
p_Verrucomicrobia;c_Opitutae;o_Puniceicoccales;f_Puniceicoccaceae;g_MB11C04	0.011%	0.018%	0.075%
p_Verrucomicrobia;c_Opitutae;o_Pelagicoccales;f_Pelagicoccaceae;g_Pelagicoccus	0.006%	0.009%	0.057%
p_Verrucomicrobia;c_Verruco-5;o_R76-B128;f_;g_	0.016%	0.016%	0.036%
p_Verrucomicrobia;c_Verruco-5;o_WCHB1-41;f_;g_	0.005%	0.007%	0.001%
p_Verrucomicrobia;c_Verruco-5;o_WCHB1-41;f_RFP12;g_	0.003%	0.005%	0.002%
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_	0.366%	0.287%	0.386%
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_Akkermansia	0.011%	0.018%	0.020%
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_Luteolibacter	0.088%	0.076%	0.024%
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_MSBL3	0.014%	0.017%	0.039%
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_Persicirhabdus	0.006%	0.006%	0.003%
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_Rubritalea	0.030%	0.017%	0.061%
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales;f_Verrucomicrobiaceae;g_Verrucomicrobium	0.007%	0.003%	0.002%
p_Verrucomicrobia;c_Pedosphaerae;o_;f_;g_	0.040%	0.035%	0.118%
p_Verrucomicrobia;c_Pedosphaerae;o_Arctic97B-4;f_;g_	0.018%	0.012%	0.051%
p_Verrucomicrobia;c_Pedosphaerae;o_Pedosphaerales;NA;NA	0.004%	0.005%	0.002%
p_Verrucomicrobia;c_Pedosphaerae;o_Pedosphaerales;f_;g_	0.010%	0.004%	0.017%
p_Verrucomicrobia;c_Pedosphaerae;o_Pedosphaerales;f_auto67_4W;g_	0.010%	0.007%	0.005%
p_Verrucomicrobia;c_Spartobacteria;o_Chthoniobacterales;f_Chthoniobacteraceae;g_	0.001%	0.005%	0.004%
p_Verrucomicrobia;c_Spartobacteria;o_Chthoniobacterales;f_Chthoniobacteraceae;g_DA101	0.017%	0.011%	0.008%
p_WPS-2;c_;o_;f_;g_	0.001%	0.007%	0.005%
p_WS3;c_PRR-12;o_GN03;f_KSB4;g_	0.004%	0.008%	0.004%
p_ZB3;c_BS119;o_;f_;g_	0.019%	0.010%	0.018%

Supplementary 7: Core-OTUs per water mass. A) Venn diagram Classification showing the per water mass core-OTUs, defined as OTUs present in 95% of samples throughout the year. B) Core-OTUs presence in the different water masses: The first column shows the taxonomic classification for each core-OTU, the second column indicate the type of distribution of each core-OTU (ubiquitous, pan or unique) and the third column indicate the water masses where the core-OTU were found.

A)



B)

Names	total	OTU_taxonomy
U30 U33 U35	60	EU799838.1.1420;p_Proteobacteria;c_Alphaproteobacteria
		AY697868.1.1486;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Sediminicola
		KC836073.1.1491;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Candidatus_Aquiluna;s_rubra
		JN625594.1.1411;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Polaribacter
		EU799638.1.1351;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
		KC001593.1.1290;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		JX984082.1.1228;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
		NCROTU175814;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		NROTU202;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
		KC250891.1.1313;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Polaribacter
		HQ672155.1.1438;p_Cyanobacteria;c_Synechococcophycideae;o_Synechococcales;f_Synechococcaceae;g_Synechococcus
		FJ825947.1.1482;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		FJ825937.1.1478;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		HQ672156.1.1493;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
		EU801074.1.1425;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_RS62
		FR684046.1.1434;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
		FJ825893.1.1472;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		JN594650.1.1328;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		HQ242270.1.1225;p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_OCS155
		JX016272.1.1492;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_HTCC2207
		NROTU192;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
		HQ241995.1.1360;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
		HQ242139.1.1428;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		EU259798.1.1444;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		NROTU386;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Candidatus_Aquiluna;s_rubra
		KC425564.1.1450;p_Proteobacteria;c_Alphaproteobacteria
		AB022713.1.1308;p_Proteobacteria;c_Gammaproteobacteria;o_HTCC2188;f_HTCC2089
		CU467449.60.1378;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae

		JQ194898.1.1346;p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_OCS155
		EU800350.1.1365;p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Pelagibacteraceae
		NCROTU147636;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		M59063.1.1411;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		EU799999.1.1337;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
		FJ664800.1.1394;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
		AACY020463337.2.1288;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		HQ224976.1.1496;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_HTCC2207
		NROTU1196;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Sediminicola
		HM591357.1.1493;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Glaciecola
		NROTU339;p_Proteobacteria;c_Alphaproteobacteria
		FJ745214.1.1374;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		EU268101.1.1448;p_Proteobacteria;c_Alphaproteobacteria
		KC593296.1.1255;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Tenacibaculum
		JN625620.1.1413;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		EU799254.1.1481;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
		EU801358.1.1484;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		NCROTU298973;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
		DQ395997.1.1462;p_Proteobacteria;c_Alphaproteobacteria;o_Sphingomonadales
		FR684119.1.1469;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae;g_Candidatus_Portiera
		AF324539.1.1486;p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
		JN625593.1.1395;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		FR683203.1.1468;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		NROTU1086;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
		HQ672205.1.1461;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		AY697917.1.1435;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
		EU803121.1.1436;p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Pelagibacteraceae
		EF572367.1.1451;p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Pelagibacteraceae
		AM110980.1.1496;p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Pseudoalteromonadaceae;g_Pseudoalteromonas
		AY794142.1.1465;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales
		JQ195283.1.1359;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
		EU268107.1.1427;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Glaciecola
U30 U33	**16**	FR684306.1.1501;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		NROTU1124;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae
		FJ172065.1.1479;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Candidatus_Aquiluna;s_rubra
		NROTU1128;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		NROTU1235;p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae
		NROTU262;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		NCROTU466776;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Roseivivax
		KF500636.1.1445;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Anaerospora
		NROTU952;p_Proteobacteria;c_Betaproteobacteria
		NROTU158;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		DQ234158.1.1560;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
		NROTU976;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae
		NROTU615;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_RS62
		DQ415790.1.1470;p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Helicobacteraceae
		EU801515.1.1502;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		HM127132.1.1445;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae
U30 U35	**7**	JX525506.1.1377;p_Proteobacteria;c_Alphaproteobacteria
		FN563281.1.1451;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_Bacteroides
		EF468717.1.1487;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
		AB301901.1.903;p_Euryarchaeota;c_Thermoplasmata;o_E2;f_Marine_group_II
		JQ196429.1.1318;p_Proteobacteria;c_Alphaproteobacteria
		KC872822.1.1407;p_Proteobacteria;c_Alphaproteobacteria
		GU119348.1.1446;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
U33 U35	**8**	EU259792.1.1496;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae;g_Candidatus_Portiera
		FR684182.1.1481;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
		JQ226602.1.1297;p_Euryarchaeota;c_Thermoplasmata;o_E2;f_Marine_group_II
		GU940734.1.1314;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae;g_Fluviicola
		EF379568.1.1502;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
		GU061120.1.1385;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		NROTU1267;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
		DQ234154.1.1513;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
U30	**13**	NCROTU452830;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_RS62
		NROTU949;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		NCROTU375849;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_RS62
		NCROTU20103;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		CU919511.1.1285;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Rhodobacter
		NCROTU97102;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Marivita
		NCROTU571682;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		JX223276.1.1471;p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae;g_Dechloromonas
		NROTU1145;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		EU801187.1.1507;p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;f_Methylophilaceae
		CU923598.1.1358;p_Proteobacteria;c_Gammaproteobacteria;o_Pseudomonadales;f_Moraxellaceae;g_Acinetobacter
		FJ745213.1.1376;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		NROTU861;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
U33	**15**	NROTU343;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Agrococcus;s_jenensis
		JN625554.1.1336;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		DQ223020.1.1315;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Pseudoruegeria
		HM127673.1.1452;p_Bacteroidetes;c_Sphingobacteriia;o_Sphingobacteriales
		NROTU1256;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Sediminicola
		NROTU126;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae
		FR684954.1.1497;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae

		NROTU154;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
		GU061244.1.1415;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Candidatus_Aquiluna;s_rubra
		NROTU582;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Polaribacter
		FJ826059.1.1477;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		EU592391.1.1393;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
		JN625561.1.1390;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		NCROTU89607;p_Proteobacteria;c_Betaproteobacteria;o_Rhodocyclales;f_Rhodocyclaceae
		AACY020294209.1241.2637;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
U35	**44**	KC899248.1.1329;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Glaciecola
		JQ197126.1.1360;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
		EU803077.1.1263;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Halomonadaceae;g_Candidatus_Portiera
		NROTU197;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Anaerospora
		KF771472.1.1426;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		NCROTU83041;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Oleispira
		GQ348037.1.1385;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
		JN015211.1.1452;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
		HQ439527.1.1426;p_Proteobacteria;c_Gammaproteobacteria;o_Vibrionales;f_Vibrionaceae
		AACY023703203.94.1439;p_Proteobacteria;c_Betaproteobacteria;o_Methylophilales;f_Methylophilaceae
		KF465366.1.1353;p_Proteobacteria;c_Epsilonproteobacteria;o_Campylobacterales;f_Campylobacteraceae;g_Arcobacter
		FJ825868.1.1491;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60
		EF010981.1.1437;p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Aeromonadaceae
		EU799369.1.1274;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
		AM117931.1.1486;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
		FJ745185.1.1370;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		EU919854.1.1457;p_Proteobacteria;c_Alphaproteobacteria
		JX527226.1.1431;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
		EU799965.1.1501;p_Proteobacteria;c_Betaproteobacteria
		JQ200226.1.1360;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
		FR684154.1.1447;p_Proteobacteria;c_Alphaproteobacteria
		FJ744882.1.1365;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Cryomorphaceae
		FJ826243.1.1483;p_Proteobacteria;c_Gammaproteobacteria;o_Thiotrichales;f_Piscirickettsiaceae
		DQ270698.1.1405;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Alcanivoracaceae;g_Alcanivorax
		NCROTU32927;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Glaciecola
		EU799386.1.1375;p_Proteobacteria;c_Alphaproteobacteria
		JQ579843.1.1483;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae
		JQ196242.1.1362;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales
		JX537881.1.1376;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Glaciecola
		HQ703826.1.1411;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oleiphilaceae
		FJ745251.1.1308;p_Proteobacteria;c_Alphaproteobacteria
		GU235798.1.1298;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Loktanella
		FO203512.3716623.3718149;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae;g_Oleispira
		NROTU1197;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_HTCC2188;g_HTCC
		EU802598.1.1454;p_Proteobacteria;c_Alphaproteobacteria
		GU235687.1.1314;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		HQ242629.1.1447;p_Proteobacteria;c_Alphaproteobacteria;o_Rickettsiales;f_Pelagibacteraceae
		KC139302.1.1245;p_Proteobacteria;c_Gammaproteobacteria;o_Oceanospirillales;f_Oceanospirillaceae
		NROTU1072;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae;g_Octadecabacter
		EU801694.1.1511;p_Bacteroidetes;c_Flavobacteriia;o_Flavobacteriales;f_Flavobacteriaceae;g_Flavobacterium
		NROTU716;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_Alteromonadaceae;g_Glaciecola
		NROTU1231;p_Actinobacteria;c_Acidimicrobiia;o_Acidimicrobiales;f_OCS155
		NROTU89;p_Proteobacteria;c_Alphaproteobacteria;o_Rhodobacterales;f_Rhodobacteraceae
		EU010228.1.1359;p_Proteobacteria;c_Gammaproteobacteria;o_Alteromonadales;f_OM60

Chapter 2:

Unraveling the environmental and anthropogenic drivers of bacterial community changes in the estuary of Bilbao and its tributaries

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Abstract

In this study, 16S rRNA gene sequencing was used to characterize the changes in taxonomic composition and environmental factors significantly influencing bacterial community structure across an annual cycle in the Estuary of Bilbao as well as its tributaries. In spite of this estuary being small and characterized by a short residence time, the environmental factors most highly correlated with the bacterial community mirrored those reported to govern larger estuaries, specifically salinity and temperature. Additionally, bacterial community changes in the estuary appeared to vary with precipitation. For example, an increase in freshwater bacteria (*Comamonadaceae* and *Sphingobacteriaceae*) was observed in high precipitation periods compared to the predominately marine-like bacteria (*Rhodobacterales* and *Oceanospirillales*) that wwere found in low precipitation periods. Notably, we observed a significantly higher relative abundance of *Comamonadaceae* than previously described in other estuaries. Furthermore, anthropic factors could have an impact on this particular estuary's bacterial community structure. For example, ecosystem changes related to the channelization of the estuary likely induced a low dissolved oxygen (DO) concentration, high temperature, and high chlorophyll concentration period in the inner euhaline water in summer (samples with salinity >30 ppt). Those samples were characterized by a high abundance of facultative anaerobes. For instance, OTUs classified as *Cryomorphaceae* and *Candidatus Aquiluna rubra* were negatively associated with DO concentration, while *Oleiphilaceae* was positively associated with DO concentration. Additionally, microorganisms related to biological treatment of wastewater (e.g *Bdellovibrio* and *Zoogloea)* were detected in the samples immediately downstream of the Bilbao Wastewater Treatment Plant (WWTP). There are several human activities planned in the region surrounding the Estuary of Bilbao (e.g. sediment draining, architectural changes, etc.) which will likely affect this ecosystem. Therefore, the addition of bacterial community profiling and diversity analysis into the estuary's ongoing monitoring program would provide a more comprehensive view of the ecological status of the Estuary of Bilbao.

Introduction

Estuaries are one of the most dynamic, complex, and species-rich ecosystems [1] primarily due to their tributaries' discharges [2]. Nutrient input from tributaries convert estuaries into highly productive environments with tide-dynamics that cause a net movement of organic matter and other nutrients from the estuary to the sea [3]. Furthermore, physicochemical parameters - salinity, temperature, pH, and dissolved oxygen (DO) concentration - are highly variable within estuaries due to the effect of freshwater input from rivers [4] and generally show a defined seasonality [5–7].

Bacteria are highly sensitive to physicochemical fluctuations [8]. To elaborate, the microbial networks of an estuary are involved in multiple diverse physicochemical processes, such as the carbon and energy pathways whereby bacteria use energy in non-living detrital carbon stores to produce microbial biomass, the chelation of metal [9], and the various processes crucial to microbiological responses to pollution. Thus, analysis of bacteria could become an important component of biological monitoring programs for the evaluation of estuarine water quality [10,11].

Urban estuaries are typical targets for water monitoring, because they fall under considerable anthropogenic pressure [12] due to their proximity to cities and harbors. The Estuary of Bilbao, the focus of this study, has suffered structural modifications in that large-scale reclamation of intertidal areas reduced the original, expansive estuary to a simple tidal channel in the mid-19th century [12]. This channelization altered the water circulation and turnover patterns in the Estuary of Bilbao, which modified both the abiotic and biotic processes as well as the seasonal patterns in the plankton community [13]. Furthermore, since the beginning of the industrial age, wastes from the city and factories have impacted the Estuary of Bilbao's ecosystem [14]. In spite of the estuary currently undergoing a recovery program that started in 1992 [14,15], there are still metal and hydrocarbon pollution remnants in the sediments and water. However, the reduction in polluting industries and the progressive implementation of an integrated sewage treatment plant have

started the process to water quality [15]. In order to survey the changes in estuarine quality, a monitoring program that began in 1989 has been examining the physicochemical variables in the water and sediments, as well as observing the fish and phyto-zooplankton communities by traditional taxonomic methods [16,17]. Besides, bacteria populations respond rapidly in terms of diversity, physiology and functional characteristics to environmental changes, and thus, the evaluation of their diversity changes and community structure could be suitable to evaluate anthropic impact. In addition, they are the base of the food web (i.e. photosynthetic bacteria) [9] with phytoplankton [18], however, they have not been studied yet in this estuary and are not included in the monitoring program. New technological advances, such as the development of Next Generation Sequencing (NGS) and its application in environmental samples, have made it possible to address this lack of data on the microbial community in high resolution, bringing forth the opportunity to incorporate this information into monitoring programs in hopes of achieving a more comprehensive view of ecosystems.

The use of amplicon sequencing to depict the makeup of bacterial communities in urban estuaries has been primarily focused on analyzing large estuaries with a well-defined salinity gradient (mixed or partially-mixed) like that of the Mississippi River in the United States [19], Sydney in Australia [20], and Kalama in Greece [21]. Although these estuaries are composed of dissimilar communities, common patterns can be observed throughout that point to salinity and temperature as main variables defining bacterial community changes in these large estuaries. In addition, significant shifts in community structure and composition have been linked to several climatological causes (rainfalls, spring tides, etc.). However, small estuaries such as the Estuary of Bilbao are characterized by a notably shorter water residence time and salinity gradient compared to those of large estuaries. The estuary's unique characteristics cause freshwater to flow through the upper water mass without mixing with the deeper saline waters, becoming brackish upon entering the sea. It had yet to be tested whether the environmental features governing bacterial changes in large estuaries are also main factors influencing the community of small estuaries.

In an attempt to define the key determinants that drive microbial ecology fluctuations in the Estuary of Bilbao, the present study characterized the bacterial communities along the salinity gradient of the small estuary and its tributaries through 16S rRNA amplicon sequencing. A longitudinal sampling and analysis of the bacterial community was conducted to unravel the ecosystems changes along an annual cycle. We differentiated the effects of seasonal periods on the estuarine bacterial community and oceanic upper layers. Specifically, we focused on bacterial changes in the inner zone of the estuary in the summer months, as previous studies have reported that the water is eutrophicated in that area during the summer due to the channelization of this estuary [13,15,22]. Moreover, in order to further expound the putative effect of additional anthropogenic pressure in the estuarine microbial communities, we analyzed the impact of the Wastewater Treatment Plant (WWTP) on the Galindo River - a tributary of the Estuary of Bilbao. To this effect, previous studies have reported that the biological reactors of WWTP allow the proliferation of certain bacteria that enable the degradation of compounds in the wastewater [23]. Those microorganisms can have a significant impact on estuarine bacterial communities [24,25]. In summary, this is the first study that surveys the microbiome of a small estuary once highly polluted by industrialization - the Estuary of Bilbao. The spatio-temporal analysis conducted in this estuary will allow elucidation of the environmental parameters most responsible for its microbial community shifts.

Material and methods

Study area

The Estuary of Bilbao - the last track of the Nervion-Ibaizabal River - is a small macro-mesotidal system located on the Basque coast, north of the Iberian Peninsula, on the Cantabrian Sea coast (43º19'N 3º1'W). The Estuary of Bilbao crosses Bilbao's metropolitan zone (~1 million inhabitants) and the center of the Biscay industrial area. The estuary is a narrow (50 - 2980 m wide) and shallow (6 - 30 m deep) channel that is 20 kilometers long. It was one of the most contaminated regions in Europe until the late 1980's and has since undergone a water recovery program in attempts to remedy the damage [15]. Except during short periods of increased river discharge, euhaline waters (salinity >30 ppt) dominate within the estuary [26]. The Estuary of Bilbao is partially mixed in the outer portions and highly stratified within the inner half - freshwater isolated in the upper layers while the bottom layers remain euhaline [22]. This stratification is a consequence of channeling the original estuary [12], which caused the freshwater to begin flowing solely through the upper stratum bypassing the bottom saline water.

The Estuary of Bilbao has several tributaries: Nervion-Ibaizabal, Kadagua, Asua, Galindo, and Gobela. These tributaries flow through a variety of areas with diverse environmental characteristics. Among them, the Nervion-Ibaizabal River has the highest discharge into the estuary, representing 66% of the estuarine waters and a 1.900 square-kilometer basin. Of note, the Galindo River is exposed to a Waste Water Treatment Plant (WWTP) and the metallurgical industry, thus input from those establishments influence the entire ecosystem. The WWTP has an average daily effluent flow of 4,000 l / sec (According to the data provided by the WWTP, http://www.bizkaia21.eus/atalak/TerritorioSostenible/Lugares/datos.asp?id=3&IdPagina=36&idioma=ca). The Galindo river is a low flowing river, its discharge never reaches more than 3,000 l / sec (except in large rainfalls, Uriarte et al, 2014). In summer its

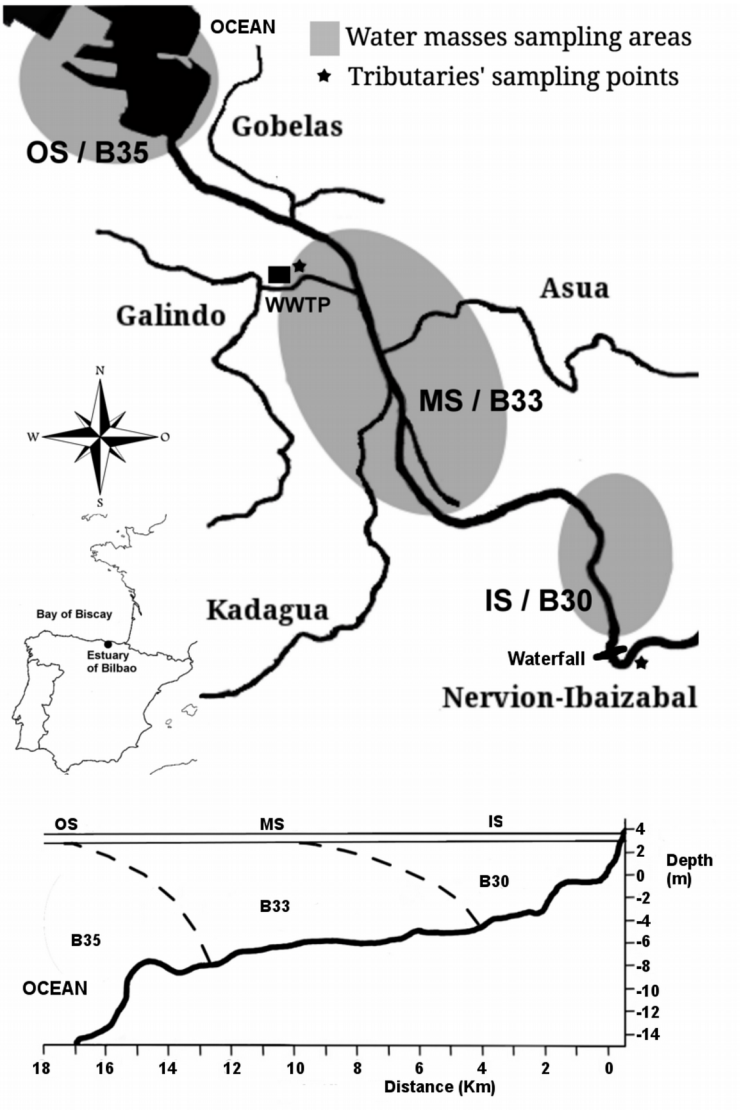
discharge barely reaches 500 l / sec. For this reason, most of the Galindo river water can have its origin in the WWTP.

Sample collection and physical and chemical analyses

Tributary samples were collected during the months of April, August and October 2014. A total of 12 samples (including two replicates) were collected in the last stretch of each tributary and from fixed points (primarily at bridges), avoiding areas affected by the tide. In the case of the Galindo River, the sampling station was 5 meters from the outlet of the WWTP.

For the estuary samples, collection was carried out monthly from August 2013 to October 2014. In total, 171 samples (including two replicates) were collected for the 14-month period. Sampling took place only on days of neap tide coefficient (30-50), always at high tide, and at approximately the same time of day (10:00 A.M.-12:00 P.M.) to eliminate confounding variables. Salinity gradient points of 30, 33 and 35 ppt were localized along the estuary each month. Once the water mass stabilized, samples were collected at a middle depth (>3 m), below the halocline (B30, B33, B35), and at the upper layer of each euhaline water mass (surface samples: IS, MS, OS, respectively) (Fig. 1). These six types of samples were collected at the estuary: 1) samples collected at salinity 30 ppt water mass. These waters are located in the inner zone of the estuary B30; 2) samples collected at the surface of B30, called IS for Inner Surface; 3) Samples collected at salinity 33 ppt, typically located in the intermediate zone of the estuary B33; 4) samples collected at the surface of B33, called MS for Middle Surface; 5) Samples collected at salinity 35 ppt, typically located in the outer most edges of the estuary B35; 6) samples collected at the surface of B35, called OS for Outer Surface (Fig. 1).

Fig. 1. Map of the Estuary of Bilbao and its tributaries. Samples were collected at two tributaries in April, August, and October 2014 [Nervion-Ibaizabal (NER) and Galindo (GAL)] indicated by stars in the figure. In addition, samples were collected at the estuary, each month from August 2013 to October 2014, indicated in grey.



Samples were collected using an oceanographic Niskin bottle. The water (10 L approx.) was stored in opaque plastic jerry cans in the field. Once in the laboratory, the water was filtered (5 L approx.) through 20 μm Nylon net filters (Millipore, 90 mm diameter) and bacteria were collected with 0.22 μm Durapore® membrane filters (Millipore, 47 mm diameter). Filtration was performed in triplicate

using a Kitasato Flask and a vacuum pump. The whole process, from sampling to storage, took less than 3 hours to perform. All filters were store at -80 ºC until DNA extraction.

At each sampling point vertical profiles (every 0.5 m) of salinity, temperature, pH, and dissolved oxygen (DO) saturation (%) were obtained in situ using a YSI 556 MPS Multiparameter Probe. Water transparency was measured with a Secchi Disk. Chl-a concentrations were calculated from spectrophotometric measurements on acetone extracts using a monochromatic method with acidification [27]. In addition, precipitation data was obtained through the Hydrometeorology Service of the Regional Council of Bizkaia (http://www.bizkaia.eus/Ingurugiroa_Lurraldea/Hidrologia_Ac/Datos_meteo.asp?Idioma=CA&Tem_Codigo=2679).

DNA extraction

Complete genomic DNA was extracted from the half of the 0.22 µm Durapore® membrane filters using PowerSoil DNA isolation kit (Mo Bio laboratories, Inc., Carlsbad, CA, USA) following the manufacturer protocol. The DNA quantity and quality of each sample was assessed by either a ND-1000 spectrophotometer (NanoDrop) or Qubit fluorimeter (Life technologies). To avoid cross-contamination all tools were flame-sterilized between samples and lab surfaces were decontaminated with DNA-ExitusPlus (Applychem) after each session. Finally, the DNA extractions were stored at -20 ºC until DNA sequencing.

16s rRNA amplification and sequencing

The 16S rRNA samples were amplified and sequenced by the Next Generation Sequencing Core at Argonne National Laboratory, Lemont, IL (USA) (http://www.earthmicrobiome.org/). Earth Microbiome Project's [28] protocols [29] were followed for the amplification and sequencing of the

community 16S v4 region by using 515f and 806r primers that contained 12 bp barcodes for sequencing. The sequencing was carried out in two MiSeq runs (2x150 paired-end). The data is available in the QIITA portal (ID 10470) and on the ENA database (study: PRJEB14094).

Bioinformatic pipeline

The raw sequences were trimmed using Sickle tool (v1.33) [30] with default parameters (including Phred score ≥ 20). Next, Pear software (v0.9.6) [31] was used to merge Illumina paired-end reads, using a cut-off of 0.01 (P-value) for the observed expected alignment score. Next, we utilized fastq-barcode.pl [32] to remove non-existent barcodes from the fastq achieved by Pear. Before carrying out the taxonomic assignment, the chimera sequences were removed by identify_chimeric_seqs.py in QIIME using the usearch61 (v7.0.1090) de novo method [33,34]. The resulting dataset was then analyzed by QIIME software (v1.9: [35]). We only included sequences that were 240-260 bp in length (average 253bp) to avoid background noise in the subsequent analyses. An open reference OTU picking method was used in QIIME for clustering using a 97% similarity cut-off using UCLUST algorithm (v1.2.22q) [33] and the taxonomy of the reference sequences was assigned based on Silva 119 database version [36] (clustered at 97% identity). The OTUs with which representative sequences failed in PYNAST alignment were discarded. After the taxonomical assignment, all chloroplast were removed from the BIOM file using filter_taxa_from_otu_table.py script in QIIME. Afterwards, samples with less than 5000 sequences were eliminated. Then, all OTUs with less than 10 sequences were removed. Finally, the BIOM file was normalized using metagenomeSeq's CSS algorithm, which normalized sequences using the cumulative sum scaling transformation [37].

Community composition analysis

First, alpha diversity analysis and taxonomic assignment were conducted to determine the community richness and composition. The alpha diversity (observed OTUs and Shannon) of the samples were calculated using phyloseq (v1.14) R package [38]. To visualize the bacterial community composition of the samples, taxa_summary_through_plot.py command in QIIME v1.9 software [35] was used.

In order to determine water mass specific OTUs as well as the OTUs that were present in all water masses, the core microbiome was analysed using compute_core_microbiome.py command on QIIME v1.9 [35]. The core microbial communities were defined as the OTUs that were present in 100% of the samples within each water mass (IS, MS, OS, B30, B33, B35) throughout the year. To compare the shared OTUs between the different water masses, a Venn diagram tool was used (http://bioinformatics.psb.ugent.be/webtools/Venn/).

A supervised learning analysis was performed for estuarine water masses using the Random Forests classifier, ten-fold cross-validation models, [39,40] and 1,000 trees. OTUs were considered “predictors” and sample type or water mass were the “class label”. This method determines the diagnostic power of bacterial profiles for predicting the characteristic community of the water masses by using a subset of samples to train a model that identifies unique features within data categories. The technique then determines the accuracy of the model by categorizing sample subsets that were not used to build the model. Through this method, we were able to evaluate not only the discriminative power in the microbial community to distinguish those groupings (sample type and water mass) but also the robustness of the groupings themselves. Therefore, the discriminative power of the microbial community in each water mass and the robustness of the groupings themselves were both evaluated for accuracy. Analysis of Similarity (ANOSIM) statistics

(999 permutations) were carried out with the ANOSIM function [41] and were used to test whether grouping samples by salinity was significant.

Principal coordinate analysis (PCoA) plots [42] were used to examine community dissimilarity and determine the impact of environmental factors (salinity, temperature, pH, DO concentration, precipitation, Chl-a) on microbial community structure. Result visualizations were made using EMPeror tool [43]. Beta diversity was estimated using the unweighted UniFrac metric for 16S rRNA amplicon data. Also, an Unweighted Pair Group Method with Arithmetic mean (UPGMA) was used to construct a tree from the unweighted UniFrac beta diversity distance matrix. This analysis aimed to characterize the differences in phylogenetic community structure.

To calculate correlations between OTUs abundances and environmental parameters, Spearman's rank correlation coefficient (rho) was carried out, by which it was possible to identify which OTUs were related to different environmental variables - salinity, temperature, pH, DO concentration, water turbidity, precipitation and chlorophyll. The impact of these environmental factors on bacterial communities was analyzed using the bio-env method of vegan (v. 2.3-4) R package [41]. This method generates Spearman’s rank correlations between the community distance matrix and an euclidean environmental distance matrix and then ranks all environmental variables by their importance. In order to calculate the percentage of beta diversity variation in each water mass explained by precipitation, an analysis of Adonis was performed.

Finally, to understand the bacterial dynamics in the inner euhaline zone of the Estuary of Bilbao, where the low DO concentrations and high values of temperature and chlorophyll concentrations dominate in summer, we used extended Local Similarity Analysis (eLSA) software [44]. The analysis was performed using OTUs with highest abundance values at B30 samples. Following eLSA software guidelines, a total of 85 OTUs were included in the analysis. To adapt to the algorithm limitations and minimize computational cost, eLSA was used to reveal statistically

significant local and potentially time-delayed association patterns between OTUs and environmental factors. Normalization of variables was performed by ‘robustZ’ method, including 14 time spots for the total number of sampling months. The rest of the analysis settings were set to default. Lastly, q-values were calculated to determine false-discovery rates. Correlations with $q<0.01$ were visualized in Cytoscape v3.2.1 [45], creating a continuous mapping-based network.

To identify the differences in OTU composition between water masses, a Kruskal-Wallis non-parametric test was carried out between tributaries and estuarine water masses. In this way, the OTUs whose abundances significantly differed between water masses were identified. Moreover, the community dissimilarity within the estuary and its tributaries were determined using a Detrended Correspondence Analysis (DCA) carried out by phyloseq (v. 1.14) R package [38].

Results

The physico-chemical analysis showed that each of the observed water masses had individual dynamics throughout the year. For instance, euhaline masses (B30, B33, B35) show little annual variation in their physico-chemical parameters, while surface waters located in the lower half of the estuary (MS, OS) showed high variability (Tables 1 and S1 Table). Furthermore, the inner euhaline zone (B30) was characterized by high turbidity and low DO saturation (Table 1). When analyzing the interaction among physicochemical factors, a negative correlation between temperature and precipitation was observed for all water masses (S2 File). In addition, temperature and salinity were negatively correlated at surface waters in the lower half of the estuary (MS, OS) while a negative correlation between temperature and DO saturation was evidenced in B30 (S2 File).

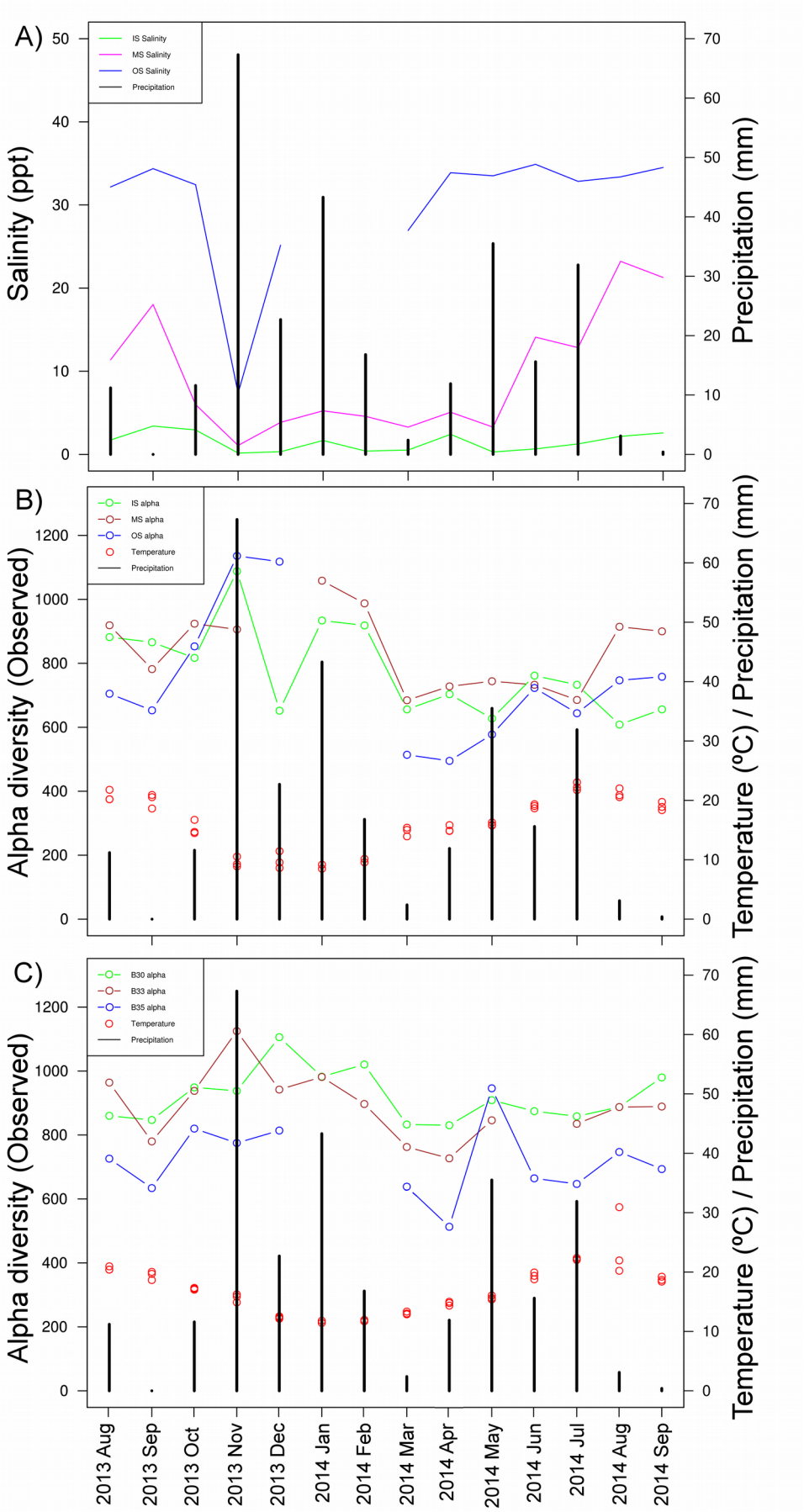
Water Mass (Nº samples)	1/Turbidity (m)	Salinity (ppt)	Temp (ºC)	DO (%)	pH	ChlA (ug/l)
IS (N=14)	NA	1.085 ± 1.496	14.757 ± 4.841	100.91 ± 12.657	8.157 ± 0.109	NA
MS (N=14)	NA	6.785 ± 9.755	15.306 ± 5.178	99.552 ± 17.418	8.086 ± 0.107	NA
OS (N=12)	NA	28.17 ± 7.675	16.457 ± 3.648	115.283 ± 30.831	8.035 ± 0.146	NA
B30 (N=14)	0.919 ± 0.779	29.905 ± 0.372	16.406 ± 3.926	36.454 ± 27.921	7.872 ± 0.152	1.331 ± 4.821
B33 (N=14)	1.347 ± 0.709	32.929 ± 0.225	16.347 ± 3.553	87.467 ± 10.588	8.01 ± 0.082	1.341 ± 1.564
B35 (N=12)	3.903 ± 2.220	34.781 ± 0.306	17.073 ± 2.966	104.773 ± 3.936	8.091 ± 0.102	0.910 ± 1.720
NER (N=3)	NA	0.347 ± 0.081	17.877 ± 3.784	99.367 ± 3.383	8.203 ± 0.182	NA
GAL (N=3)	NA	8.423 ± 12.033	20.493 ± 2.353	76.3 ± 12.692	7.59 ± 0.560	NA

Table 1. Annual Mean and Standard Deviation values of physico-chemical parameters for each water mass. The annual values represented in the table were calculated based on the monthly measurements. Raw data are detailed in the S1 Table. The environmental parameters

measured are: Turbidity (measured by Secchi disk depth), Salinity, Temperature (ºC), Dissolved Oxygen (DO) saturation, pH, and Chlorophyll (ChlA).

With regard to bacterial community analysis, the high-throughput sequencing approach yielded a total number of 3.98 millions of 16S rRNA sequences in the 155 samples collected in this study after eliminating OTUs assigned to chloroplasts (13% of the reads), ones appearing in less than 10 reads, and samples with less than 5000 reads. The alpha diversity values of each water mass oscillate throughout the year. The major diversity changes were observed in the surface waters (IS, MS, OS) in the dates with greater precipitations (Fig. 2). Precipitation causes the surface waters of the lower half of the estuary (MS and OS) to become brackish (5-30 ppt) from December to May, which increases community richness (Fig. 2).

Fig. 2. Main environmental features variations (temperature, salinity and precipitation) and community richness changes along the annual cycle. A) Monthly salinity and precipitation variation per surface water mass IS, MS, OS; B) Observed alpha diversity, temperature and precipitation fluctuation per surface water mass IS, MS, OS; C) Observed alpha diversity, temperature and precipitation changes per euhaline water mass B30, B33, B35.

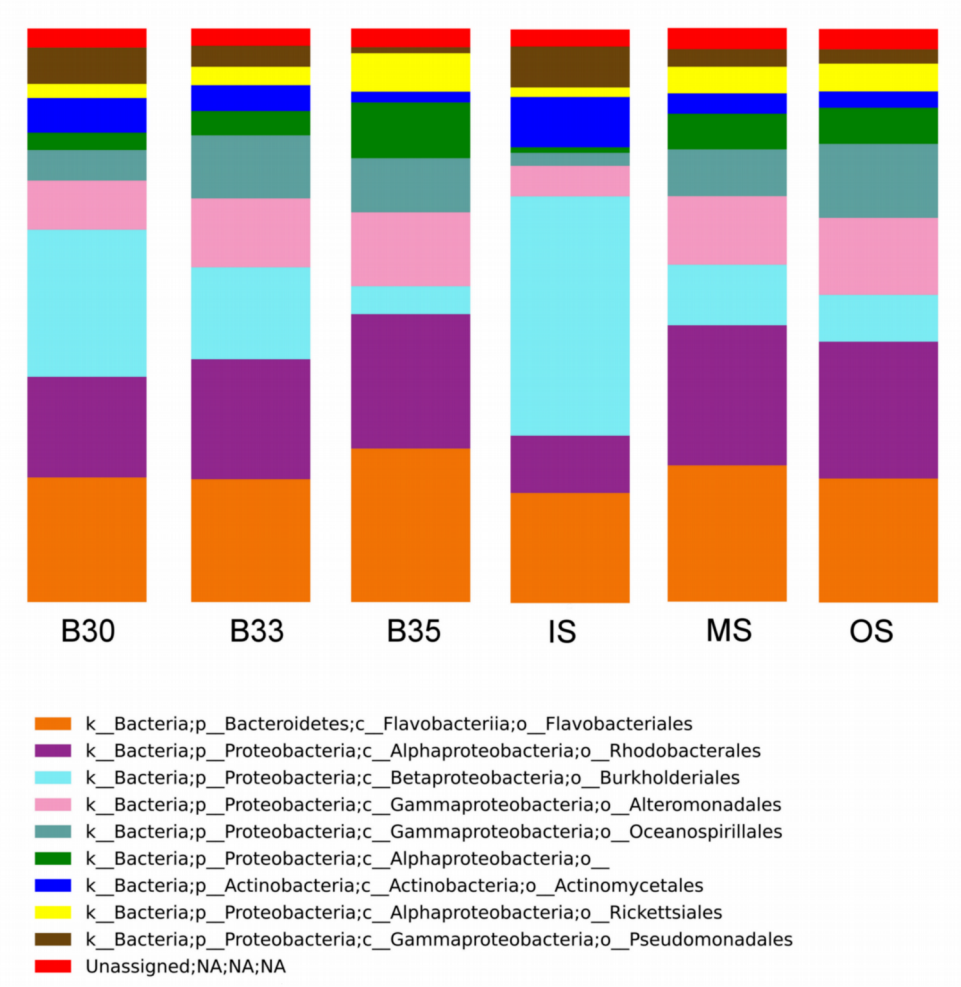

A)
IS Salinity
MS Salinity
OS Salinity
Precipitation
Salinity (ppt)
Precipitation (mm)
B)
IS alpha
MS alpha
OS alpha
Temperature
Precipitation
Alpha diversity (Observed)
Temperature (ºC) / Precipitation (mm)
C)
B30 alpha
B33 alpha
B35 alpha
Temperature
Precipitation
Alpha diversity (Observed)
Temperature (ºC) / Precipitation (mm)
2013 Aug
2013 Sep
2013 Oct
2013 Nov
2013 Dec
2014 Jan
2014 Feb
2014 Mar
2014 Apr
2014 May
2014 Jun
2014 Jul
2014 Aug
2014 Sep

All euhaline water masses (B30, B33 and B35) were dominated by OTUs affiliated with *Flavobacteriales* (14.3-19.6%), *Rhodobacterale*s (14-17.1%), *Alteromonadale*s (8-9.4%), *Oceanospirillales* (7.4%), and *Burkholderiales* (7%) orders (Fig. 3). Nevertheless, small but significant differences were found between euhaline samples (Anosim, $R < 0.19$, $p < 0.034$). In contrast, significantly high Anosim R-values were evidenced between surface waters (IS *vs.* OS, $R = 0.785$, $p < 0.001$). Within surface waters (IS, MS and OS), high abundances of OTUs related to *Flavobacteriales* (11.6-17.5%), *Rhodobacterales* (6-18%), *Actinomycetales* (5.3%), and *Pseudomonadales* (4.2%) were found. The *Burkholderiales* group was particularly abundant in IS (24.8%), decreasing gradually to reach a mean of 7.7% in OS (Figs 3 and S3 File). Surface waters were also characterized by high abundances of OTUs related to *Flavobacteriales* (11.6-17.5%), *Rhodobacterales* (6-18%), *Actinomycetales* (5.3%), and *Pseudomonadales* (4.2%).

The core OTU analysis (OTUs present in all the samples within each water mass during the entire cycle) showed that the euhaline samples contained the highest number of core OTUs: 94, 100 and 92 OTUs for the B30, B33 and B35 samples, respectively. Among the surface waters, IS showed the highest numbers of core OTUs (89), while the other two surface waters - MS and OS - showed fewer core OTUs (42 and 25, respectively). When analyzing the core OTUs shared amongst water masses, yearlong trends showed that the euhaline waters had a high number of shared OTUs between them (20) (S4 File). Interestingly, IS had the highest number of unique OTUs (50) while MS and OS had the lowest unique OTUs values (6 and 0, respectively). Random Forests model results evidenced a high classification error for MS and OS samples (0.35 and 0.6667 class error, respectively). Conversely, samples from IS, B30, B33 and B35 had a lower than 0.12 classification error (Table 2).

Fig. 3. Microbial community composition in the water masses of the estuary of Bilbao.

OTUs relative abundances per water mass were plotted. Each column shows the mean relative abundance of the top 10 most abundant orders per water sample (B30, B33, B35, IS, MS, OS) along the annual cycle (14 months). These bacteria account for the 68% of the total community.



	Assigned to						
Origin from	IS	MS	OS	B30	B33	B35	Class error
IS	20	1	0	1	0	0	0.0909
MS	3	13	1	2	1	0	0.35
OS	1	1	5	1	2	5	0.6667
B30	0	0	0	21	2	0	0.087
B33	0	0	0	1	23	0	0.0417
B35	0	0	0	1	1	15	0.1176

Table 2. Confusion matrix for the water masses of the estuary of Bilbao A Random forests classification analysis was conducted based on the communities dissimilarities among water masses. In this confusion matrix, the first column refers to where the samples were collected, while row numbers indicate the number of samples that are predicted to belong to each water mass. The classification error value is the rate of misclassified samples within each mass.

Annual dynamics of bacterial communities

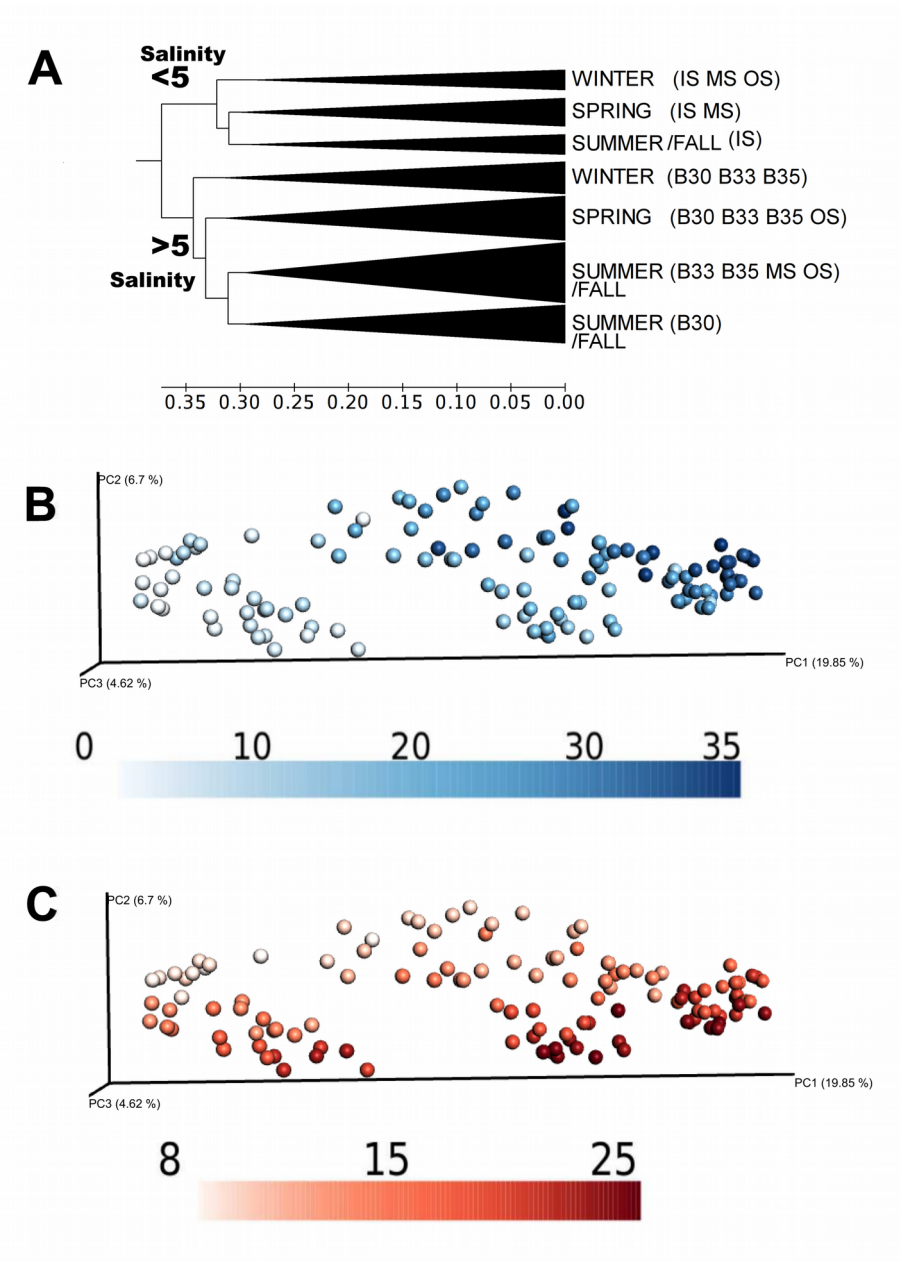
The UPGMA cluster method grouped the samples by salinity values; freshwater and saline water samples (brackish and euhaline, respectively). This result was also supported by the Spearman's correlation test, in which salinity had the highest values (rho = 0.719). Samples collected at salinities less than 5 ppt were differentiated from the rest with a beta diversity greater than 0.35 (Fig. 4). Particular taxa were found to be significantly associated to the salinity gradient (S5 Table). Spearman's analysis related some bacterial families (*Comamonadaceae*, *Oxalobacteraceae*, and *Rhodocyclaceae*) with low saline concentration waters. In the same way, the taxa related with euhaline water masses such as *Halomonadaceae*, *Piscirickettsiaceae*, and *Pelagibacteraceae* were more abundant in the B35 water mass.

Additionally, season was a secondary driver of microbiome fluctuations, whereby samples were clustered into three seasons in the UPGMA tree: winter, spring, and summer/fall with a beta diversity value greater than 0.3 (Fig. 4A). Similarly, temperature was the second strongest environmental factor varying in synchronously with bacterial community changes (Spearman rho = 0.342). This environmental variable showed a stronger correlation with surface waters communities (IS, OS, MS; rho > 0.57) than with euhaline ones (B30, B33, B35; rho < 0.46) (S6 Table). Certain OTUs were negatively related to temperature changes (*Pseudomonadaceae* and *Sphingobacteriaceae*) while other members showed a positive correlation (*Verrumicrobiaceae* and *Microbacteriaceae*) (S7 Table).

Beyond temperature, precipitation higher in winter and spring (S1 Table), seemed to reflect variations in the bacterial community makeup. The Spearman rank correlation evidenced that this feature correlation was higher for surface waters (IS, MS and OS) than for euhaline samples (S6 Table). In addition, a correlation gradient from the inner to outermost surface waters was found (rho = 0.20-0.36 in IS and OS, respectively). In rainy periods (December to May), MS and OS samples cluster within freshwater group (Fig. 4A). The composition of MS and OS in that time has an increase of freshwater bacteria (S3 File and S8 Table) such as *Burkholderiales* order, mostly *Comamonadaceae* family, and *Pseudomonadales* (S3 File). Conversely, in low precipitation periods, MS and OS grouped together with euhaline samples communities in the UPGMA tree (Fig. 4A).

Fig. 4. Dynamics and classification of bacterial communities of the estuary of Bilbao. A) UPGMA tree of the samples from the Estuary of Bilbao based on the unweighted UniFrac beta diversity distance matrix. Samples with a beta diversity distance less than 0.30 are collapsed into same branches. Seasons are defined according to the natural temporal changes in the northern hemisphere, considering winter (22 Dec-21 Mar), spring (22 Mar-21 Jun), summer (22 Jun-21 Sept) and fall (22 Sept-21 Dec). B) Unweighted UniFrac distance principal coordinate analysis

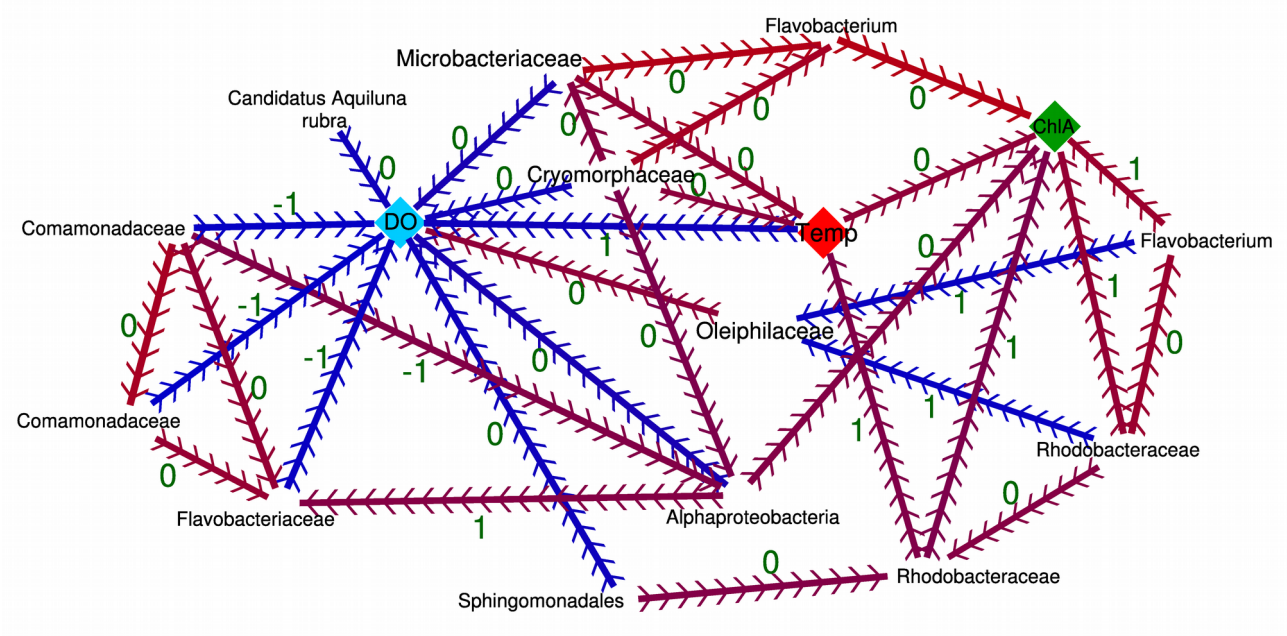
(PCoA plot) colored by salinity gradient: from freshwater (0 ppt) to saline water (35 ppt). Darker signifies a higher salinity. C) Unweighted UniFrac PCoA plot colored by temperature gradient: from low temperature (from 8ºC) to high temperature (to 25ºC). Darker signifies a higher temperature.



Among euhaline samples, B30 water mass community clustered separately from the rest in summer (Fig. 4A). The B30 water mass was characterized by high turbidity, acidic pH, high chlorophyll concentration, and low DO concentration (Table 1). For this water mass, DO

concentration combined with temperature showed a rho explanation value of 0.809. According to the extended Local Similarity Analysis (eLSA), some OTUs were related to DO concentration, temperature and chlorophyll concentration through time (Fig. 5), while no significant correlations were found for the remaining environmental variables (salinity, pH, turbidity, precipitation). *Alphaproteobacteria*, *Candidatus Aquiluna rubra*, *Comamonadaceae*, *Cryomorphaceae*, *Flavobacteriaceae*, *Microbacteriaceae,* and *Sphingomonadales* were negatively correlated with DO concentration. Specifically, *Comamonadaceae* and *Flavobacteriaceae* had a temporal delay of one month regarding DO concentration. Additionally, *Oleiphilaceae* positively correlated with DO concentration. The analysis showed that temperature and chlorophyll concentration were positively related to each other, while temperature was negatively related to DO concentration. This is also evidenced in the interaction of OTUs and environmental features, where the OTUs classified as *Rhodobacteraceae* and *Flavobacteriaceae* clades were positively related with temperature and chlorophyll concentration but not with DO concentration. Conversely, *Cryomorphaceae* and *Microbacteriaceae* were positively related to temperature but negatively with DO concentration (Fig. 5). Even though, it was not possible to assign some of the significantly related OTUs further than family rank.

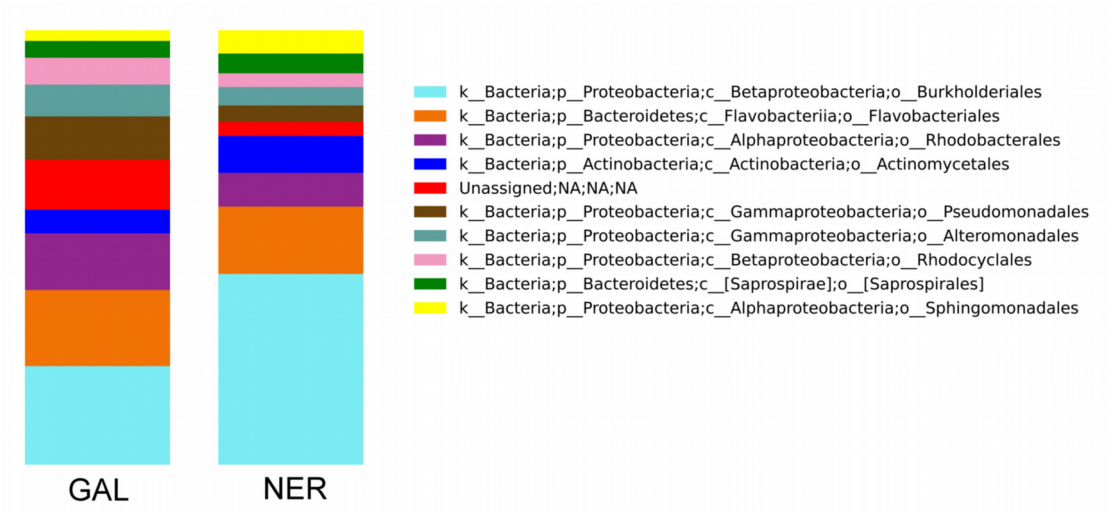
Fig. 5. The OTUs significantly related with DO concentration, temperature, and chlorophyll concentration in B30 water mass through time. eLSA analysis was conducted for the 14 time points (total sampling months in duplicate) of the B30 water mass samples. In analysis, the 85 most abundant OTUs and all the environmental features measured were included (salinity, temperature, precipitation, pH, turbidity, and chlorophyll and DO concentration). The matrix of the variables was normalized by 'robustZ' method. A network was created with Cytoscape software [45] using the significant ($q < 0.01$) correlations obtained in the eLSA analysis. The directionality of the relationship is marked with arrows with its temporal delay (in months) in the edge label (green) and the relation type between them positive (red) or negative (blue).



Tributaries

A distinguishable bacteria profile was found in the main tributaries of the Estuary of Bilbao (Fig. 6). The community of the tributary with the highest water discharge (66%), the Nervion (NER), was dominated by *Burkholderiales*, which represents up to 32% of the community. Regarding the Galindo tributary (GAL), the most abundant orders were the following: *Burkholderiales* (11.92%), *Flavobacteriales* (9.2%), *Rhodobacterales* (6.86%), and *Pseudomonadales* (5.27%). These samples were collected 5 meters downstream from a WWTP outlet and showed a unique composition of OTUs related to *Sulfurimonas*, *Bdellovibrio,* and *Zoogloea* genus (S8 Table).

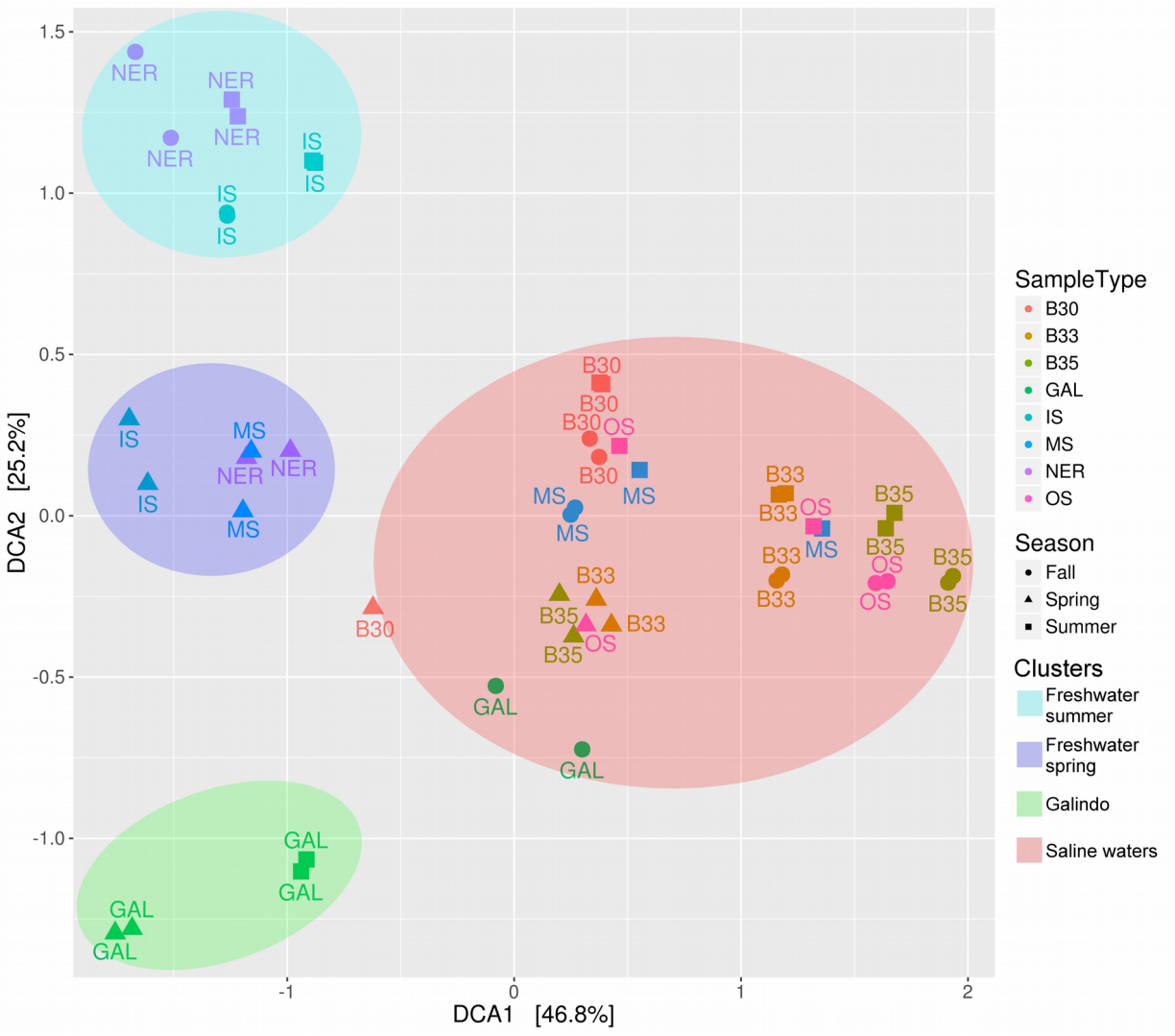
Fig. 6. Microbial taxonomic composition of the Estuary of Bilbao tributaries Nervion (NER) and Galindo (GAL). In the bar-plot, each column shows the mean relative abundances of the top 10 most abundant orders in each tributary in April, August, and October 2014. The taxonomic groups represented in the plot account for 63% of the total community.



When analyzing the samples from the Estuary of Bilbao together with the two tributaries, the communities were grouped first by salinity and then by season (Fig. 7), driven by precipitation and

temperature changes. Freshwater masses (IS, NER, GAL) were further divided into three clusters: A) Galindo samples' community that clustered apart, B) freshwater spring and C) freshwater summer (Fig. 7). However, Galindo's samples during fall were influenced by brackish water (salinity = 22 ppt) and so those bacterial communities clustered with saline water samples.

Fig. 7. Bacterial community distribution for estuary and tributary samples. A Bray-Curtis DCA plot showing the community dissimilarity among estuarine water samples (IS, MS, OS, B30, B33, B35) and the samples of the two tributary stations (GAL, NER) collected in April, August, and October 2014.



Discussion

Expanding upon the knowledge about bacterial community´s cycles in highly dynamic ecosystems, such as estuaries under the influence of the mixed effect of tides, strong salinity gradients, and sporadic flood events, should lead to a better understanding of the adaptability of microorganisms to different physicochemical changes [3,8,12]. Furthermore, regular monitoring of bacterial diversity changes along environmental and pollution gradients would distinguish factors that influence estuarine ecosystem variation. Recent developments in DNA sequencing techniques allow screening taxonomic diversity in water samples affordably and reliably [46]. In this regard, by applying 16S rRNA amplicon sequencing to different salinity water masses along an annual cycle, this study is the first detailed survey of bacterial diversity in the Estuary of Bilbao. The main tributaries were included in the study and several environmental parameters (such as salinity, temperature, dissolved oxygen concentration, turbidity, pH, and chlorophyll concentration) were observed to give further context to the revealed patterns. High rainfall during the winter and spring, a period of low DO concentration with high levels of chlorophyll and temperature in the inner estuary (B30) in summer [13,15,21,25], and the thermal variation happening from winter to summer - all appeared to correlate with fluctuations in the bacterial community. Despite these various microbial compositions, a similar community makeup was found at both the beginning and end of this yearlong study, suggesting an annual cycling of the microbial community.

The estuary of Bilbao: the case of a small urban estuary

The geomorphology of the Estuary of Bilbao contrasts to that of large estuaries characterized to date through amplicon sequencing (Mississippi [19], Sydney [20], etc.). The Estuary of Bilbao is short (~20 km) with low river discharge (max = 37 m^3 s^{-1}) and a contrasting tidal fluxing rate (max = 700 m^3 s^{-1} in the outer zone and max = 10 m^3 s^{-1} in the inner zone) [13]. Together, these factors

cause bacteria to have a short residence time in surface waters. However, similar to the results shown in large estuaries [7,19,20], the bacterial communities of the Estuary of Bilbao were classified in two large groups according to salinity: brackish-euhaline communities (salinity > 5 ppt) and freshwater communities (salinity < 5 ppt) (Fig. 4). Accordingly, the Spearman correlation values between OTUs and environmental features showed highest rho values when testing for salinity (rho = 0.72-0.85 for salinity and rho = 0.52-0.64 for temperature, S5 and S7 Tables). Further, within the groupings (brackish-euhaline and freshwater), samples clustered by season (Fig. 4 A), revealing temperature as a secondary driver of community drifts. This is in line with previous work showing that aside from salinity, temperature significantly influences the microbiomes of long and short estuaries, as well as estuaries under high or low anthropogenic pressures [2,4,6,20].

Outside seasonal variation, the euhaline water samples (B30, B33, B35) had a relatively stable core microbial community due to the ocean's buffer effect. These results are consistent with Chow and colleagues (2013) findings that reported little oscillation in the communities of the upper ocean layers [47]. Contrarily, estuarine surface layers waters, particularly MS and OS (Table 1 and S1 Table), showed higher variability in both environmental features and bacterial community structure (Fig. 4). Sugimoto and colleagues [48] found that in the surface waters of Ise bay in Japan, salinity and other environmental parameters (turbidity, temperature, etc.) were significantly altered by the river discharge and precipitation. Similarly in the Estuary of Bilbao, MS and OS samples become brackish during rainy periods (Fig. 2), showing a high number of freshwater-related taxa (e.g. *Burkholderiales*, S3 File) as well as an increase in their alpha diversity (Fig. 2). Accordingly, brackish waters have been considered among the richest waters in the estuary [1,7,49-52]).

In high precipitation periods, the river discharge increases and curtails the residence time of the water [13] encouraging the presence of freshwater-related taxa in the outer waters of the Estuary of Bilbao, where flow is lessened (e.g. *Burkholderiales*, S3 File). Moreover, an increase of alpha

diversity was observed during the high precipitation period (Fig. 2) in the surface waters of the lower half of the estuary (MS and OS). Similar results have been shown in previous studies (e.g. [1,7,49-52]) where brackish waters have been noted among the richest portions of an estuary with high bacterial diversity. Assumedly, precipitation and river discharge affect an estuary's brackish waters, where mixed communities comprised of bacterioplankton populations from multiple water masses are able to interact [52].

Apart from these general patterns, summer eutrophication events have been reported since the 19th century in the inner part of the urban Estuary of Bilbao following water stratification [13,15,22,26]. Similarly, summer B30 samples from this study were characterized by a severely low DO concentration with high temperatures and chlorophyll concentrations (Tables 1 and S1 Table). This situation is believed to be a consequence of the conversion of the original estuary by the large-scale reclamation of intertidal areas into a minor tidal channel [14]. As outlined by Uriarte and colleagues [13], the channelization causes an increase in water turnover time of the inner euhaline water, leading to a decrease in DO concentration that coincides with the decrease of tributary water discharge. This particular environmental situation leads to a distinct community in summer B30 euhaline samples. For instance, OTUs related to *Candidatus Aquiluna rubra* stood out as the ones with highest abundance increases during low DO concentrations. This species has been previously described and detected in eutrophic freshwater [53] and in harbors' seawater [54]. Additionally, *Comamonadaceae* family and *Flavobacterium* genus increased in abundance one month before the decrease in DO concentration (Fig. 5). Although these bacteria are known freshwater-typical aerobic organisms, they can become facultative anaerobic denitrifiers [55,56]. Thus, they could survive in anoxic waters as they perform denitrification. Several variables are interrelated in eutrophication processes [13, 15, 22, 26] (e.g. DO is negatively correlated to temperature, while the correlation between chlorophyll concentration and temperature is positive), which is evidenced further in this community (Fig. 5). Additional environmental factors that were not measured in this study, such as nutrients, wind, nitrites, nitrates, etc., may play a significant role in

defining the microbial community and would be necessary measures to construct a higher resolution interaction network. The sequencing of an additional gene would also help in a better understanding of the OTUs and environmental factors' interactions, as it would allow a better taxonomic categorization of OTUs.

Tributaries

Similarly to the estuarine communities, the bacteria communities of the tributaries were grouped by salinity (Fig. 7). However, both tributaries waters (Galindo and Nervion-Ibaizabal) each had a distinguishable community and specific physicochemical properties that characterized them.

Galindo tributary samples, which were collected downstream of a WWTP, were the most unrelated samples due to the bacteria used in the activated sludge. Within them, some OTUs belonging to *Bdellovibrio* and *Zoogloea* genera stood out. These types of bacteria proliferate in the different steps of wastewater treatment [57] and they are related with different processes of water treatment (e.g. *Bdellovibrio* as a bacterial predator). Furthermore, *Zoogloea* species are typically used in domestic and aerobic sewage-treatment systems, such as trickling filters, activated sludge tanks, or oxidation ponds [57]. In light of this, we can conclude that since the WWTP implementing a biological treatment step in 2001-2002, which dramatically reduces the contribution of the plant effluent to the river [58], the water discharge contains bacteria from the activated sludge. However, as amplicon sequencing does not distinguish between living and dead cells, it remains to be tested whether these bacteria are functionally active; thus, a shotgun or metatranscriptomics approach would be needed. In any case, these taxa were not detected in the estuarine samples (as can be seen in S8 Table), meaning that either: 1) they get diluted in the estuarine waters and a higher sequencing coverage might be needed to identify them; or 2) the low river discharge of this

tributary might not be enough to counter the tidal flux of the estuary and freshwater mass would shift upwards when the tide rises [59], making the waters stagnant in the inner Galindo's basin.

Regarding the Nervion tributary, *Comamonadaceae* - related OTUs were the most abundant family (27.3±7.5% of the bacterial community), while its abundance only represented 4.43±6.37% in outer estuarine waters (B35) (S3 File). Interestingly, *Comamonadaceae* abundances found in the Estuary of Bilbao are among the highest detected compared to other studied estuaries [7,60,61]. Within this family, the most abundant two OTUs could not be classified beyond the family taxonomic level, as shown in Fig. 5. *Comamonadaceae* has a remarkable metabolic diversity that includes aerobic organotrophs, anaerobic denitrifiers and Fe^{3+}-reducing bacteria, hydrogen oxidizers, photoautotrophic and photoheterotrophic bacteria, and fermentative bacteria [62,63].

Conclusions

In conclusion, in this preliminary survey of the bacterial diversity of the Estuary of Bilbao, the sequencing of the 16S rRNA gene showed that salinity and temperature are the most prominent abiotic factors varied synchronously with bacterial community changes in this estuary, as is the case with larger estuaries. Moreover, additional environmental factors need to be studied to acquire a more representative picture of the dynamics of the estuary's diverse water masses. For instance, precipitation and resulting river discharge is linked to the appearance of mixed communities in surface waters. Additionally, certain OTUs correlated with DO concentration, temperature, and chlorophyll concentration in the inner euhaline waters in summer expose a unique community characterized by a higher abundance of facultative anaerobic denitrifiers. The defining characteristics of each river (orography, stratigraphy, different types of anthropogenic impact) contribute different substrates to the bacterial communities of the estuary and therefore, future studies addressing these factors are recommended. Future endeavors would involve sampling a more expansive area of each river/tributary to be able to characterize the provenance of each bacterial assemblage and to indirectly monitor discharges from the different anthropogenic sources (human and industrial waste, WWTP, etc.) potentially affecting the system. Furthermore, studying the metabolic cycles of these communities via gene expression analysis (i.e. metatranscriptomics; [64]) would give further insights into the biochemical dynamics beyond taxonomy. Thereby, functional metagenomic research could improve our understanding of bacterial functions in specific biochemical cycles related to anthropogenic pressure (e.g. sulfur-reduction and denitrification processes) in these ecosystems.

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Supplementary material

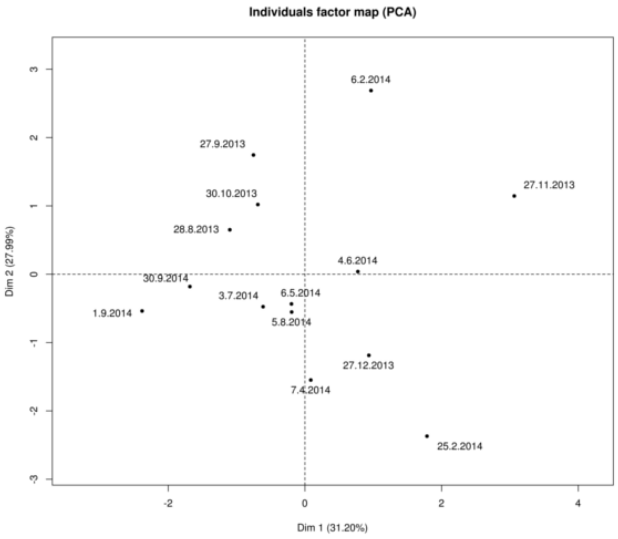
Supplementary 1: Physico-chemical values for each estuarine water mass by sampling date. Water samples were collected monthly between August 2013 - Sept. 2014 except when weather precluded it.

Water mass	Sampling date	Precipitation (mm)	Secchi (m)	Salinity (ppt)	Temperature (ºC)	DO (%)	pH	ChlA (ug/l)
IS	28.8.2013	11.20	0.65	1.73	20.20	100.30	8.07	NA
IS	27.9.2013	0.00	1.00	3.41	20.54	128.60	8.05	NA
IS	30.10.2013	11.60	0.50	2.94	14.69	99.30	8.09	NA
IS	27.11.2013	67.30	1.00	0.16	8.88	116.60	8.05	NA
IS	27.12.2013	22.70	0.20	0.32	8.62	100.80	8.33	NA
IS	6.2.2014	43.30	1.20	5.24	9.17	104.40	8.00	NA
IS	25.2.2014	16.80	3.00	0.40	9.56	106.80	8.33	NA
IS	7.4.2014	2.40	2.50	0.52	15.03	97.30	8.18	NA
IS	6.5.2014	11.90	1.50	2.41	14.89	100.20	8.22	NA
IS	4.6.2014	35.50	1.50	0.29	15.86	100.00	8.07	NA
IS	3.7.2014	15.60	0.50	0.66	18.66	99.40	8.20	NA
IS	5.8.2014	31.90	0.75	1.26	21.74	108.60	8.30	NA
IS	1.9.2014	3.10	0.75	2.17	20.87	72.70	8.16	NA
IS	30.9.2014	0.40	1.00	2.58	18.90	88.40	8.16	NA
MS	28.8.2013	11.20	1.30	11.38	21.78	NA	8.11	NA
MS	27.9.2013	0.00	1.75	18.03	20.91	128.30	8.06	NA
MS	30.10.2013	11.60	1.00	5.99	14.49	88.40	8.10	NA
MS	27.11.2013	67.30	1.00	1.07	9.27	116.30	8.05	NA
MS	27.12.2013	22.70	0.20	3.85	9.55	100.80	8.16	NA
MS	6.2.2014	43.30	1.00	1.67	8.49	106.80	8.09	NA
MS	25.2.2014	16.80	2.50	4.57	10.14	96.60	8.18	NA
MS	7.4.2014	2.40	3.00	3.28	15.40	96.30	8.23	NA
MS	6.5.2014	11.90	1.50	5.07	15.83	NA	8.16	NA
MS	4.6.2014	35.50	1.25	3.28	16.29	99.80	8.12	NA
MS	3.7.2014	15.60	1.50	32.77	19.36	84.20	7.96	NA
MS	5.8.2014	31.90	1.25	12.84	23.04	132.00	8.21	NA
MS	1.9.2014	3.10	2.00	23.22	22.02	85.20	7.89	NA
MS	30.9.2014	0.40	2.25	21.28	19.74	75.90	7.90	NA
OS	28.8.2013	11.20	3.50	32.17	20.18	NA	8.15	NA
OS	27.9.2013	0.00	5.00	34.37	18.63	202.00	8.05	NA
OS	30.10.2013	11.60	2.00	32.44	16.72	103.20	8.12	NA
OS	27.11.2013	67.30	1.50	7.37	10.53	111.40	8.11	NA
OS	27.12.2013	22.70	1.50	25.16	11.45	113.00	8.12	NA
OS	7.4.2014	2.40	9.50	26.93	13.94	104.70	7.65	NA
OS	6.5.2014	11.90	5.00	33.88	14.85	NA	7.99	NA
OS	4.6.2014	35.50	4.50	33.51	15.76	121.50	8.03	NA
OS	3.7.2014	15.60	6.00	32.46	19.06	104.90	8.06	NA
OS	5.8.2014	31.90	5.50	32.84	22.19	123.80	8.20	NA
OS	1.9.2014	3.10	5.00	33.38	20.55	97.10	7.89	NA
OS	30.9.2014	0.40	4.50	34.51	18.36	98.50	8.07	NA

Water mass	Sampling date	Precipitation (mm)	Secchi (m)	Salinity (ppt)	Temperature (ºC)	DO (%)	pH	ChlA (ug/l)
B30	28.8.2013	11.20	0.65	30.05	20.96	NA	8.03	3.83
B30	27.9.2013	0.00	1.00	30.29	20.03	79.30	8.09	1.25
B30	30.10.2013	11.60	0.50	30.18	17.28	52.10	8.00	0.77
B30	27.11.2013	67.30	1.00	30.26	14.89	51.20	7.97	0.26
B30	27.12.2013	22.70	0.20	30.31	12.14	66.80	8.03	0.58
B30	6.2.2014	43.30	1.00	30.06	11.44	90.90	7.86	0.41
B30	25.2.2014	16.80	3.00	30.14	11.70	65.00	8.05	0.27
B30	7.4.2014	2.40	2.50	29.88	12.88	65.40	7.75	1.08
B30	6.5.2014	11.90	1.50	29.33	15.00	NA	7.85	1.20
B30	4.6.2014	35.50	1.50	29.42	16.05	46.10	7.74	1.28
B30	3.7.2014	15.60	0.50	29.24	19.93	6.00	7.65	5.70
B30	5.8.2014	31.90	0.75	29.54	22.37	8.30	7.72	18.73
B30	1.9.2014	3.10	0.75	29.88	21.95	15.10	7.70	1.75
B30	30.9.2014	0.40	1.00	30.12	19.22	29.10	7.78	2.91
B33	28.8.2013	11.20	1.30	33.01	20.40	NA	8.11	1.20
B33	27.9.2013	0.00	1.75	33.13	19.70	107.00	8.11	4.48
B33	30.10.2013	11.60	1.00	33.02	17.20	107.00	8.03	0.80
B33	27.11.2013	67.30	1.00	33.09	15.87	73.20	8.09	0.47
B33	27.12.2013	22.70	0.20	32.98	12.54	87.40	8.15	0.52
B33	6.2.2014	43.30	1.20	33.10	11.79	96.40	7.89	0.38
B33	25.2.2014	16.80	2.50	32.62	11.94	77.80	8.04	0.72
B33	7.4.2014	2.40	3.00	33.06	13.02	88.10	7.93	1.05
B33	6.5.2014	11.90	1.50	32.34	14.78	NA	7.93	2.78
B33	4.6.2014	35.50	1.25	32.92	15.62	88.20	7.91	2.20
B33	3.7.2014	15.60	1.50	32.77	19.36	84.20	7.96	1.81
B33	5.8.2014	31.90	1.25	32.96	22.11	80.30	8.01	5.61
B33	1.9.2014	3.10	2.00	32.86	20.92	83.50	8.01	2.38
B33	30.9.2014	0.40	2.25	33.16	18.67	83.20	7.98	1.36
B35	28.8.2013	11.20	3.50	34.99	20.41	NA	8.16	0.47
B35	27.9.2013	0.00	5.00	34.93	18.65	104.80	8.13	2.03
B35	30.10.2013	11.60	2.00	35.03	17.33	107.40	8.12	0.29
B35	27.11.2013	67.30	1.50	34.93	16.24	96.90	8.13	0.10
B35	27.12.2013	22.70	1.50	34.99	12.65	104.00	8.22	0.36
B35	7.4.2014	2.40	9.50	34.01	13.34	104.00	7.80	0.55
B35	6.5.2014	11.90	5.00	34.95	14.34	NA	8.06	3.20
B35	4.6.2014	35.50	4.50	34.78	15.38	104.00	8.05	4.38
B35	3.7.2014	15.60	6.00	34.89	18.78	112.00	8.10	1.01
B35	5.8.2014	31.90	5.50	34.40	22.04	105.20	8.11	5.18
B35	1.9.2014	3.10	5.00	34.58	20.22	107.90	8.13	0.83
B35	30.9.2014	0.40	4.50	34.91	18.39	102.20	8.09	0.96

Supplementary 2: Within environmental variables correlations per water mass. Principal Components Analysis (PCA) plots for samples (“individual factor map”) and environmental variables distribution (“variables factor map”).

IS


Individuals factor map (PCA)
6.2.2014
27.9.2013
27.11.2013
30.10.2013
28.8.2013
4.6.2014
30.9.2014
3.7.2014
6.5.2014
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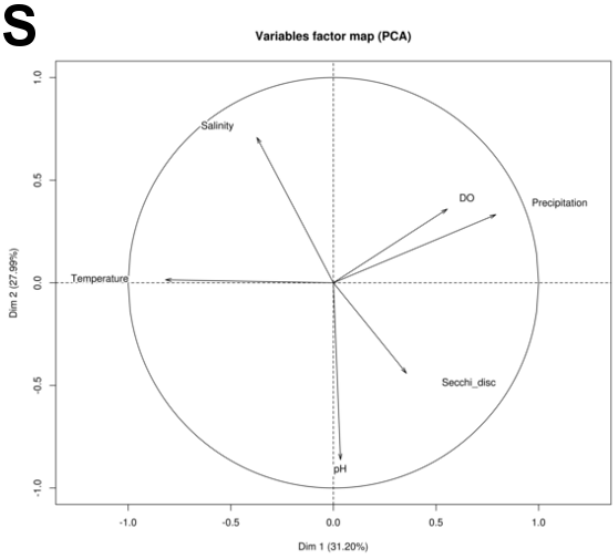
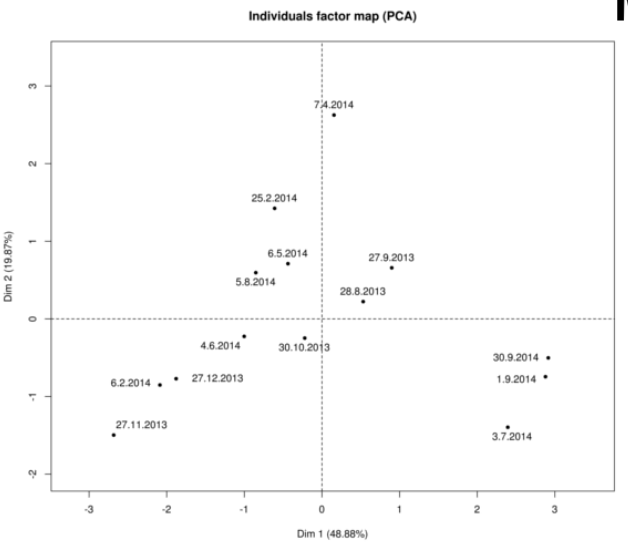
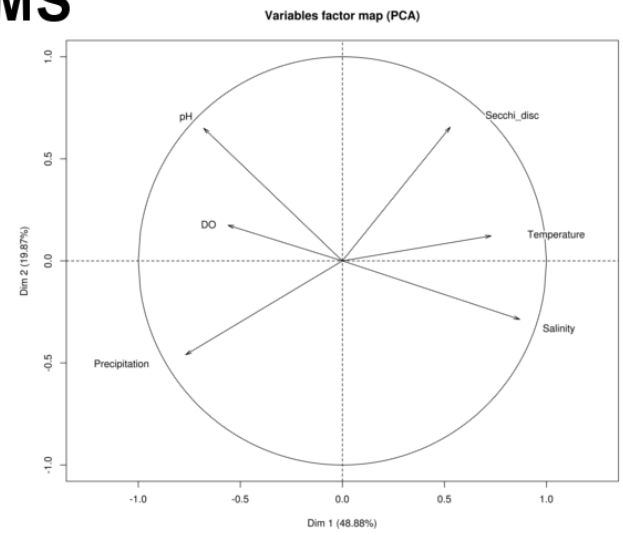
5.8.2014
27.12.2013
7.4.2014
25.2.2014
Dim 2 (27.99%)
Dim 1 (31.20%)


Variables factor map (PCA)
Salinity
DO
Precipitation
Temperature
Secchi_disc
pH
Dim 2 (27.99%)
Dim 1 (31.20%)

MS


Individuals factor map (PCA)
7.4.2014
25.2.2014
6.5.2014
27.9.2013
5.8.2014
28.8.2013
4.6.2014
30.10.2013
30.9.2014
6.2.2014
27.12.2013
1.9.2014
27.11.2013
3.7.2014
Dim 2 (19.87%)
Dim 1 (48.88%)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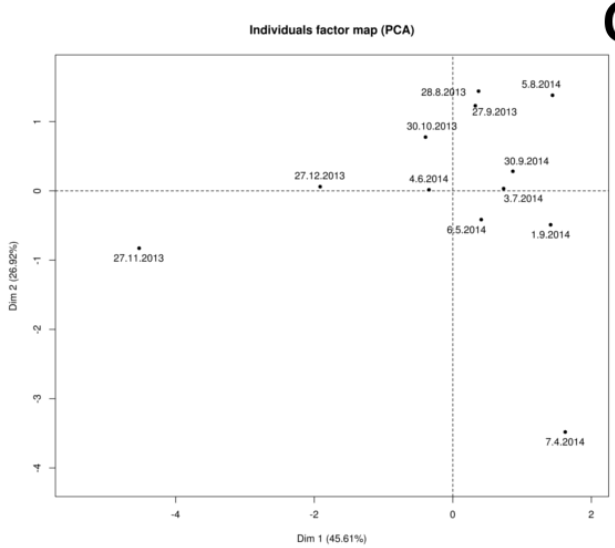
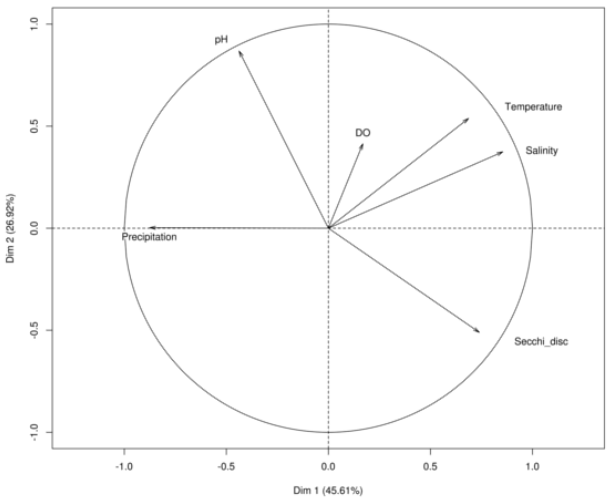
pH
Secchi_disc
DO
Temperature
Salinity
Precipitation
Dim 2 (19.87%)
Dim 1 (48.88%)

OS


Individuals factor map (PCA)
5.8.2014
28.8.2013
27.9.2013
30.10.2013
30.9.2014
27.12.2013
4.6.2014
3.7.2014
6.5.2014
1.9.2014
27.11.2013
7.4.2014
Dim 2 (26.92%)
Dim 1 (45.61%)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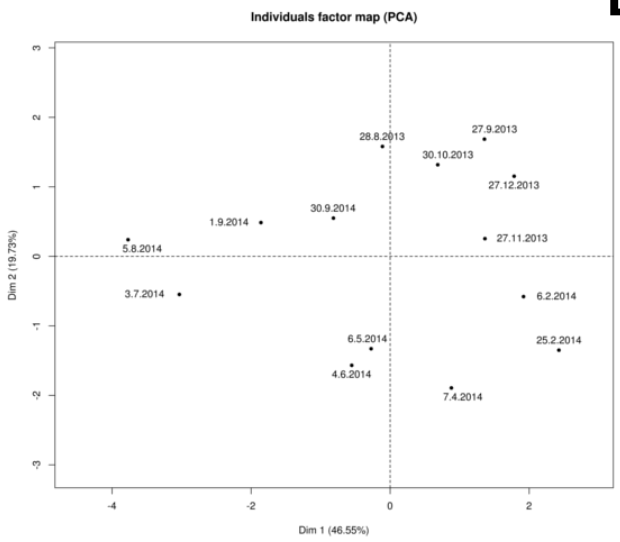
pH
Temperature
DO
Salinity
Precipitation
Secchi_disc
Dim 2 (26.92%)
Dim 1 (45.61%)

B30


Individuals factor map (PCA)
28.8.2013
27.9.2013
30.10.2013
27.12.2013
30.9.2014
1.9.2014
27.11.2013
5.8.2014
3.7.2014
6.2.2014
6.5.2014
25.2.2014
4.6.2014
7.4.2014
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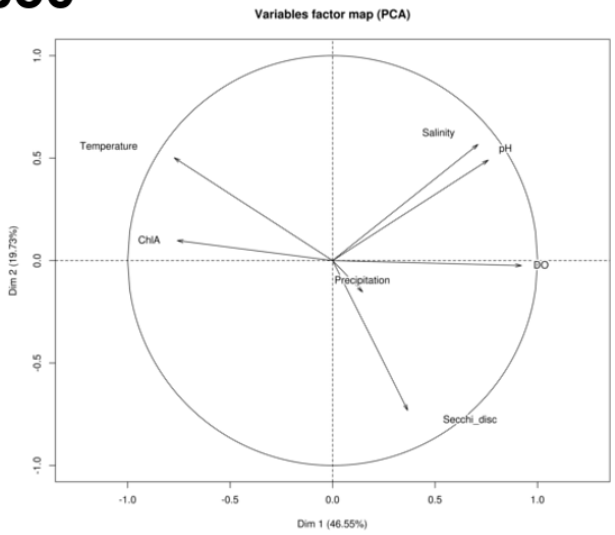
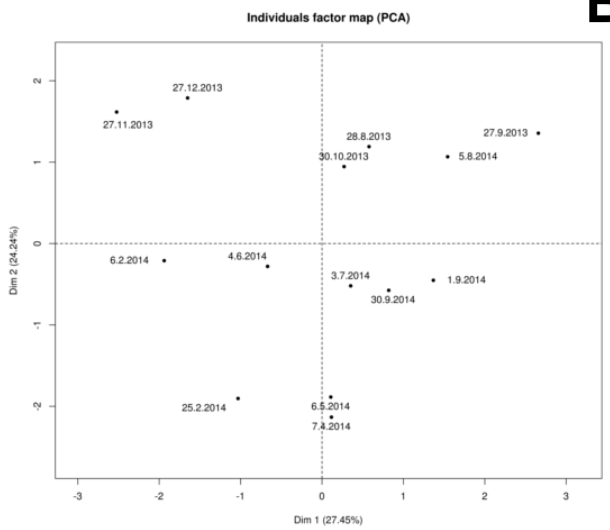
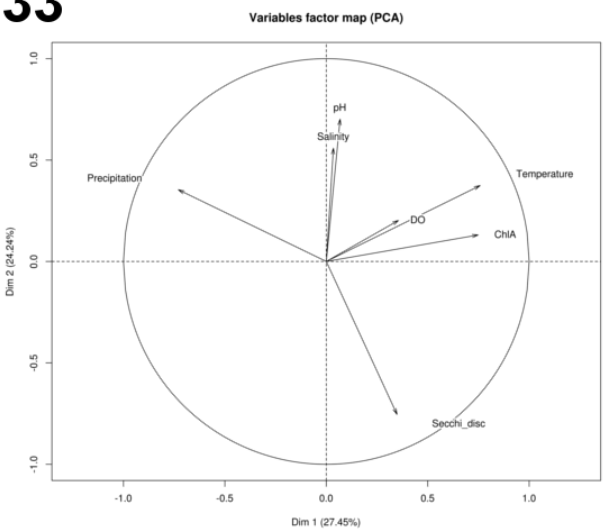
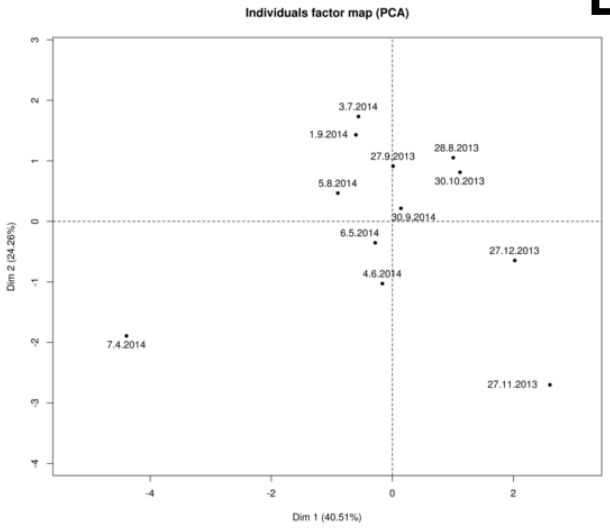

Variables factor map (PCA)
Temperature
Salinity
pH
ChlA
DO
Precipitation
Secchi_disc
Dim 2 (19.73%)
Dim 1 (46.55%)

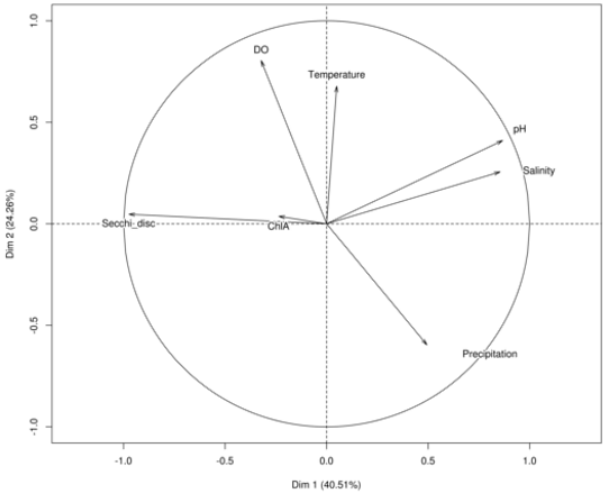
B33


Individuals factor map (PCA)
27.12.2013
27.11.2013
28.8.2013
27.9.2013
30.10.2013
5.8.2014
6.2.2014
4.6.2014
3.7.2014
1.9.2014
30.9.2014
25.2.2014
6.5.2014
7.4.2014
Dim 2 (24.24%)
Dim 1 (27.45%)


Variables factor map (PCA)
pH
Salinity
Precipitation
Temperature
DO
ChlA
Secchi_disc
Dim 2 (24.24%)
Dim 1 (27.45%)

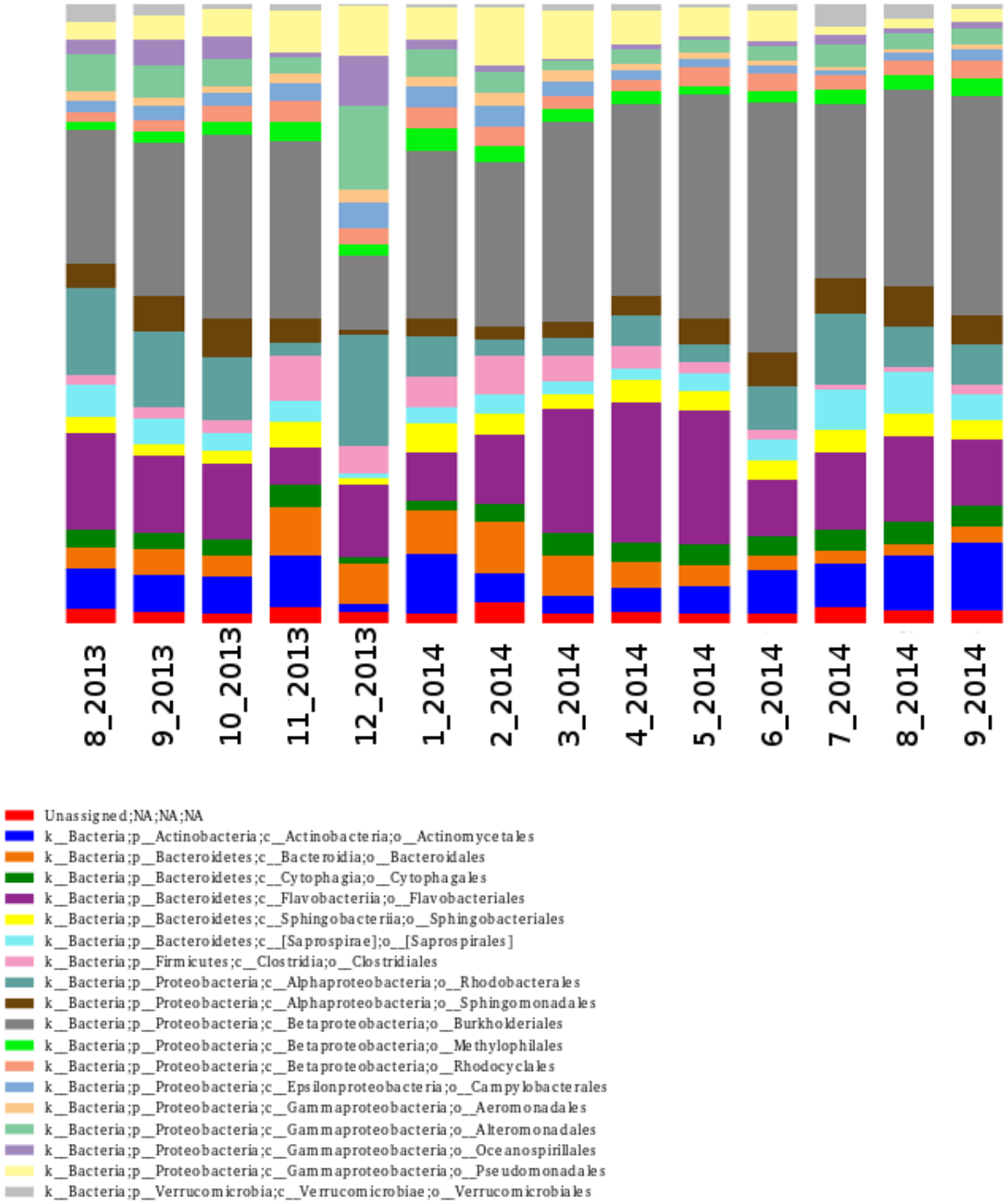
B35


Individuals factor map (PCA)
3.7.2014
1.9.2014
27.9.2013
28.8.2013
5.8.2014
30.10.2013
30.9.2014
6.5.2014
27.12.2013
4.6.2014
7.4.2014
27.11.2013
Dim 2 (24.26%)
Dim 1 (40.51%)

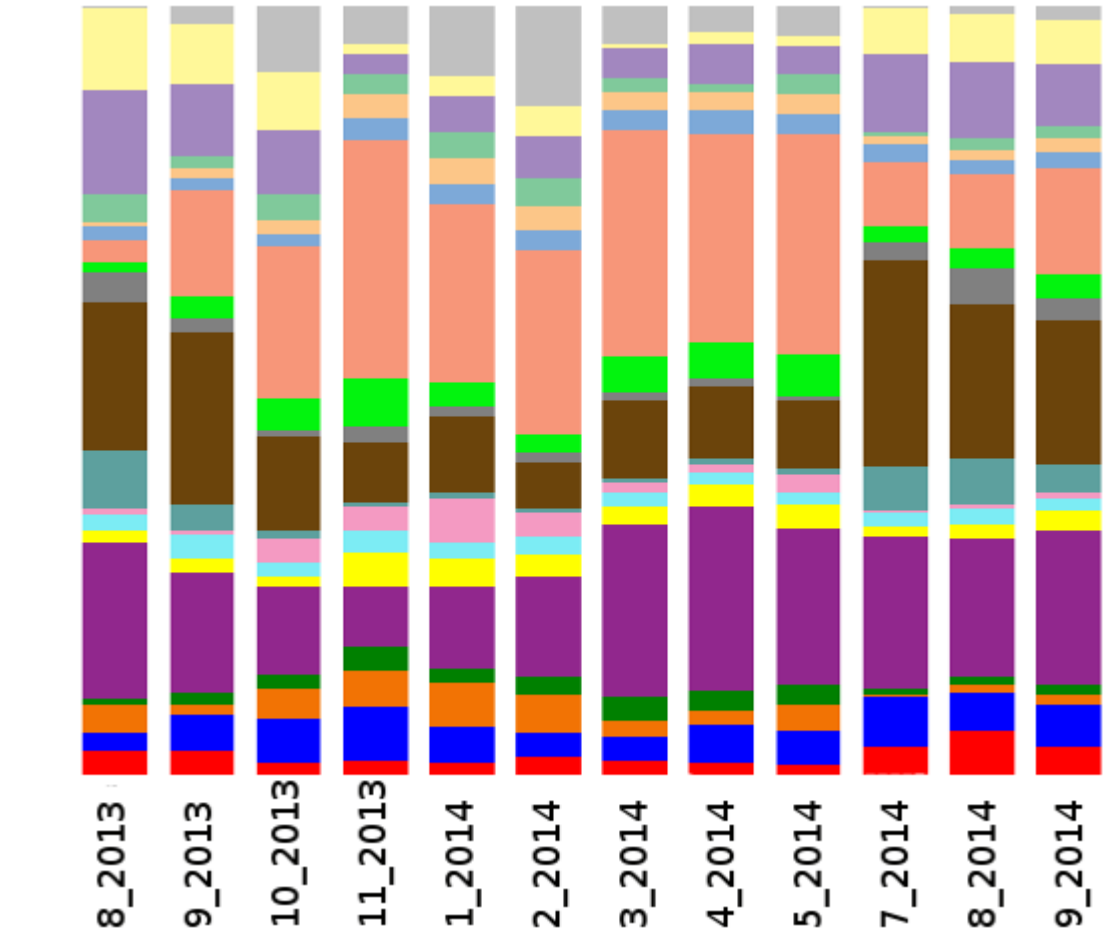

Variables factor map (PCA)
DO
Temperature
pH
Salinity
Secchi_disc
ChlA
Precipitation
Dim 2 (24.26%)
Dim 1 (40.51%)

Supplementary 3: Community changes along the annual cycle per estuarine water mass. Taxonomy barplots show orders with greater abundance than 1%. The labels indicate the collection date of each sample.

IS water mass



MS water mass



Unassigned;NA;NA;NA
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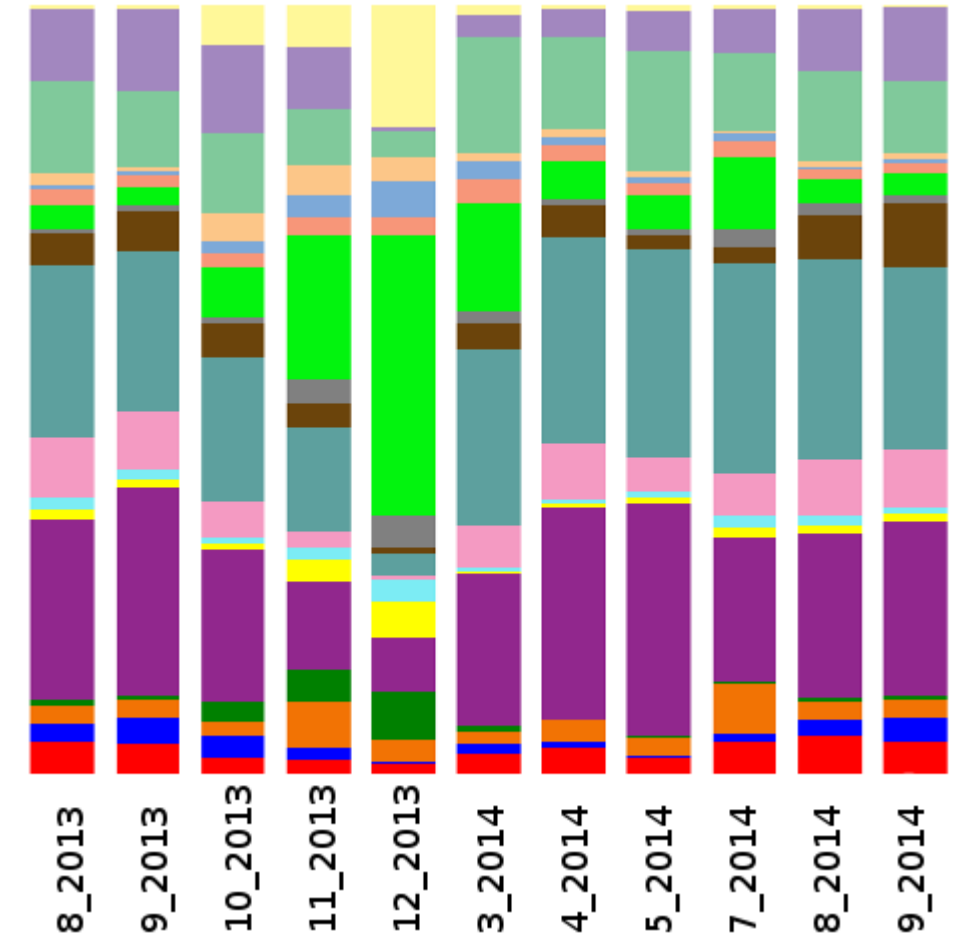
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales]
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales

OS water mass



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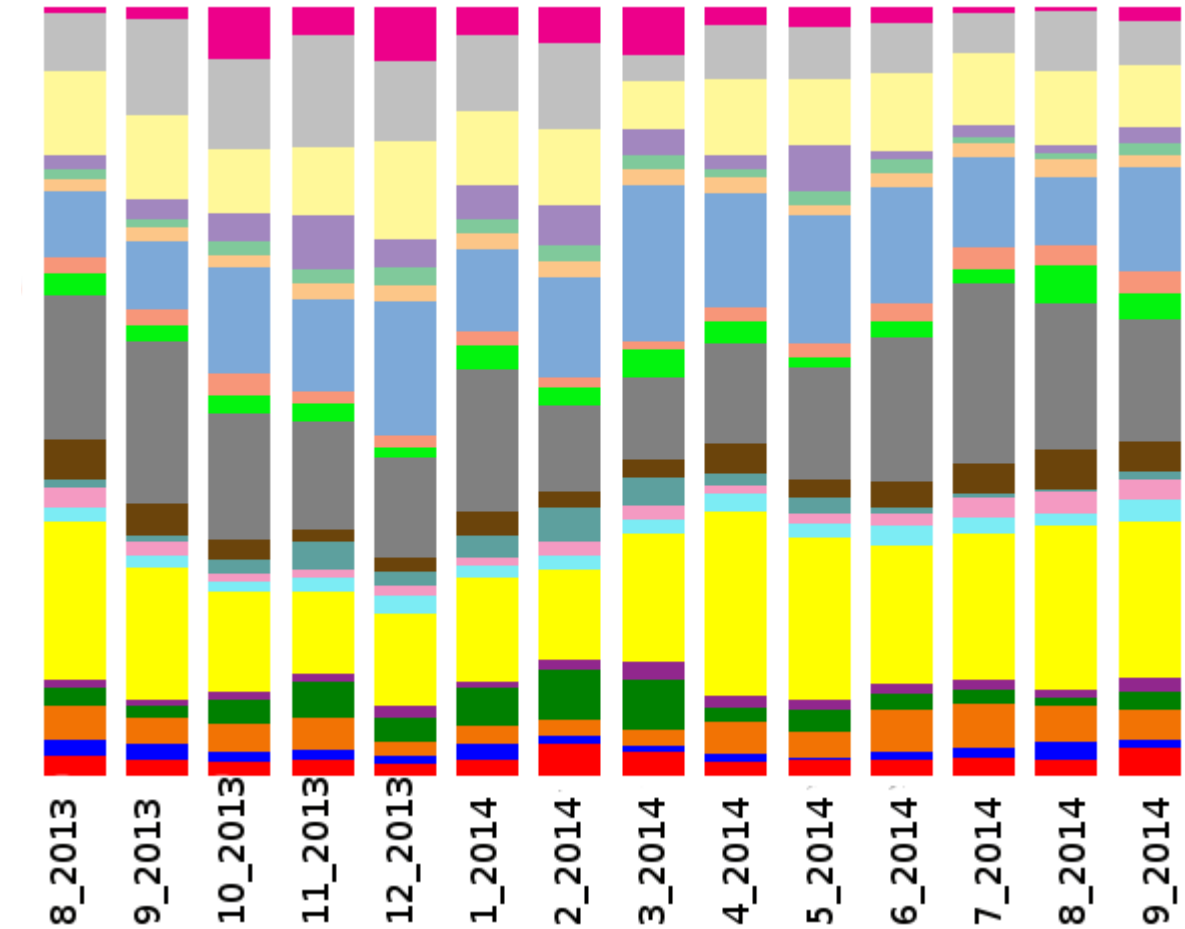
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales]
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales

B30 water mass



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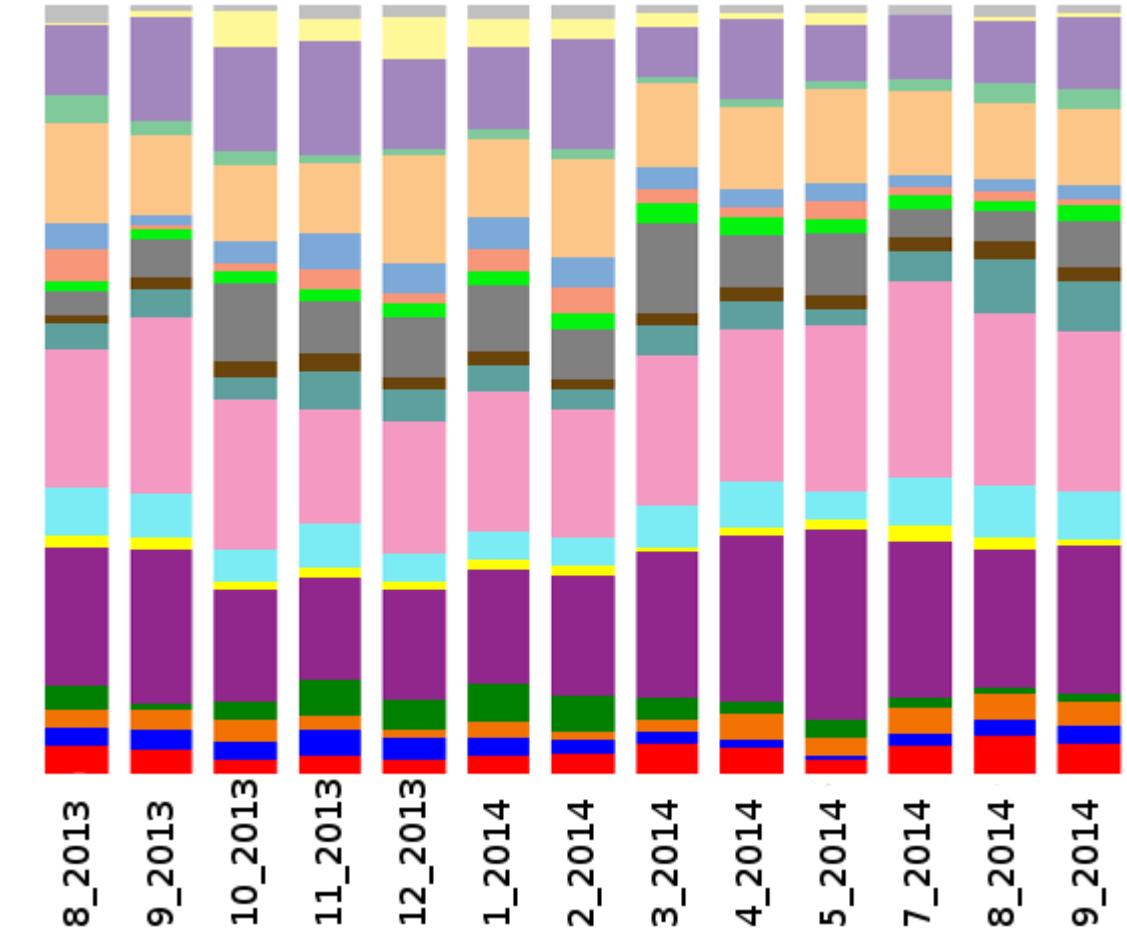
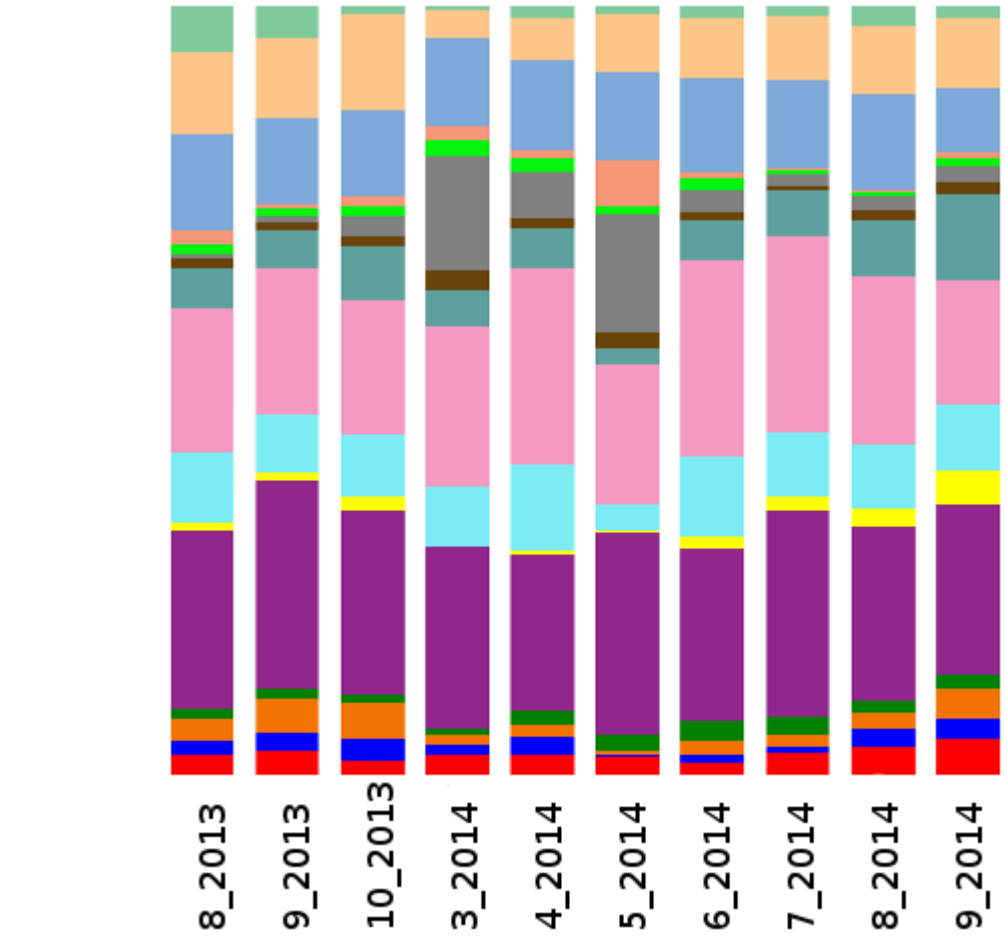
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales]
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales

B33 water mass



B35 water mass



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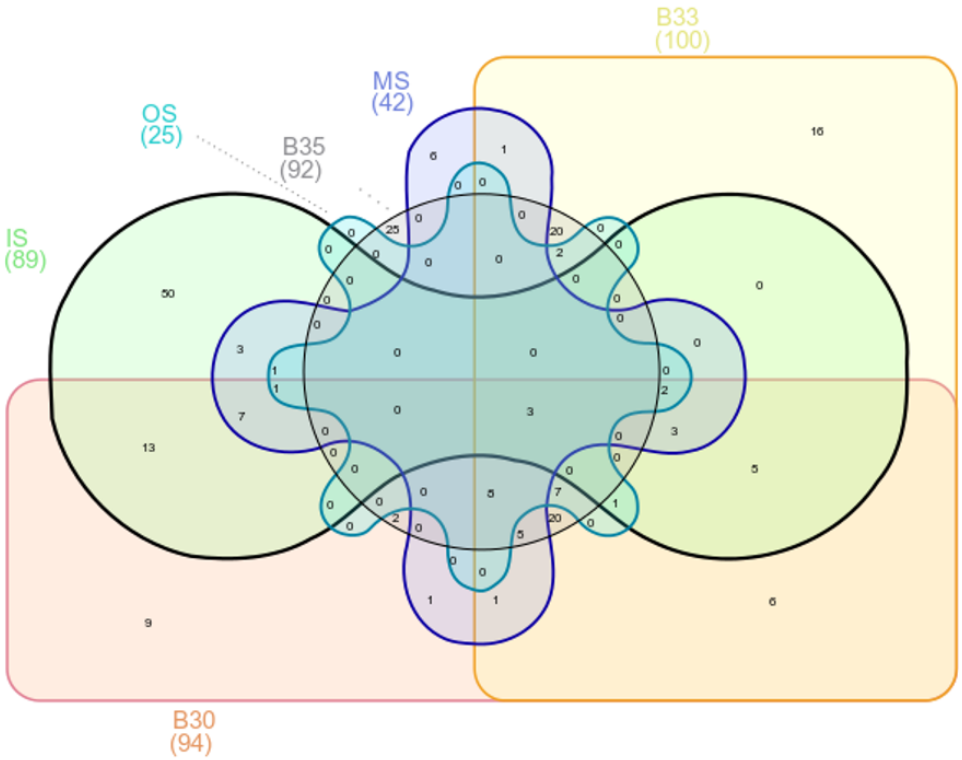
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales
k__Bacteria;p__Cyanobacteria;c__Synechococcophycideae;o__Synechococcales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Methylophilales
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales

Supplementary 4: Core-OTUs per water mass. A) Venn diagram Classification showing the per water mass core-OTUs, defined as OTUs present in 100% of samples throughout the year. B) Core-OTUs presence in the different water masses: The first column shows the taxonomic classification for each core-OTU, the second column indicate the type of distribution of each core-OTU (ubiquitous, pan or unique) and the third column indicate the water masses where the core-OTU were found.

A)



B)

TAXA	TYPE	WATER MASSES
p__Proteobacteria,_f__Comamonadaceae_EU801074.1.1425	ubiquitous	IS MS B30 B33 B35 OS
p__Proteobacteria,_f__Rhodobacteraceae_EU799638.1.1351	ubiquitous	IS MS B30 B33 B35 OS
p__Proteobacteria,_f__Rhodobacteraceae_CU919511.1.1285	ubiquitous	IS MS B30 B33 B35 OS
p__Proteobacteria,_f__Campylobacteraceae_AF324539.1.1486	pan	IS MS B30 B33 OS
p__Proteobacteria,_f__Comamonadaceae_GU247463.1.1238	pan	IS MS B30 B33 OS
p__Bacteroidetes,_f__Cryomorphaceae_FJ825937.1.1478	pan	MS B30 B33 B35 OS
p__Proteobacteria,_f__HTCC2188_FJ745214.1.1374	pan	MS B30 B33 B35 OS
p__Proteobacteria,_f__Rhodobacteraceae_AY697917.1.1435	pan	MS B30 B33 B35 OS
p__Proteobacteria,_f__OM60_DQ234158.1.1560	pan	MS B30 B33 B35 OS
p__Proteobacteria,_f__Rhodobacteraceae_New.ReferenceOTU147636	pan	MS B30 B33 B35 OS
p__Proteobacteria,_f__Alteromonadaceae_HQ224976.1.1496	pan	MS B30 B33 B35 OS
p__Proteobacteria,_f__SUP05_GQ345764.1.1378	pan	MS B30 B33 B35 OS
p__Bacteroidetes,_f__Flavobacteriaceae_AY697868.1.1486	pan	MS B30 B33 B35 OS
p__Proteobacteria,_o__Sphingomonadales_New.ReferenceOTU478	pan	IS MS B30 OS
p__Proteobacteria,_f__Oxalobacteraceae_EU802044.1.1501	pan	IS MS B30 B33
p__Bacteroidetes,_f__Chitinophagaceae_EU803334.1.1400	pan	IS MS B30 B33
p__Proteobacteria,_f__Comamonadaceae_EU234180.1.1515	pan	IS MS B30 B33
p__Actinobacteria,_f__Microbacteriaceae_KC836073.1.1491	pan	IS B30 B33 OS
p__Proteobacteria,_f__Rhodobacteraceae_JX984082.1.1228	pan	MS B30 B33 B35
p__Bacteroidetes,_f__Cryomorphaceae_JN625620.1.1413	pan	MS B30 B33 B35
p__Proteobacteria,_f__Rhodobacteraceae_HQ242139.1.1428	pan	MS B30 B33 B35
p__Proteobacteria,_f__Rhodobacteraceae_FJ664800.1.1394	pan	MS B30 B33 B35
p__Proteobacteria,_f__Rhodobacteraceae_EU259798.1.1444	pan	MS B30 B33 B35
p__Proteobacteria,_f__Halomonadaceae_FR684119.1.1469	pan	B30 B33 B35 OS
p__Actinobacteria,_f__OCS155_JQ194898.1.1346	pan	B30 B33 B35 OS
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p__Proteobacteria,_c__Alphaproteobacteria_New.ReferenceOTU339	pan	B30 B33 B35 OS
p__Bacteroidetes,_f__Cryomorphaceae_FJ825893.1.1472	pan	B30 B33 B35 OS
p__Proteobacteria,_c__Alphaproteobacteria_EU799838.1.1420	pan	B30 B33 B35 OS
p__Actinobacteria,_f__OCS155_HQ242270.1.1225	pan	B30 B33 B35 OS
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p__Actinobacteria,_f__Microbacteriaceae_HM127132.1.1445	pan	IS MS B30
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p__Proteobacteria,_f__Rhodobacteraceae_CU920376.2.1285	pan	IS MS B30
p__Proteobacteria,_f__Methylophilaceae_EU801622.1.1502	pan	IS MS B30
p__Proteobacteria,_f__Comamonadaceae_JQ965782.1.1392	pan	IS MS B30
p__Proteobacteria,_f__Aeromonadaceae_EF010981.1.1437	pan	IS B30 B33
p__Proteobacteria,_f__Comamonadaceae_AM778025.1.1612	pan	IS B30 B33
p__Proteobacteria,_f__Comamonadaceae_HE589809.1.1451	pan	IS B30 B33
p__Bacteroidetes,_f__Cytophagaceae_EU801738.1.1482	pan	IS B30 B33
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p__Bacteroidetes,_f__Flavobacteriaceae_JX527226.1.1431	pan	B30 B33 B35
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p__Bacteroidetes,_f__Cryomorphaceae_EU801358.1.1484	unique	B30
p__Proteobacteria,_f__Comamonadaceae_New.ReferenceOTU745	unique	B30
p__Proteobacteria,_f__Comamonadaceae_CU920682.1.1366	unique	B30
p__Proteobacteria,_c__Alphaproteobacteria_New.ReferenceOTU1059	unique	B30
p__Bacteroidetes,_f__Cryomorphaceae_JN625593.1.1395	unique	B30
p__Proteobacteria,_c__Betaproteobacteria_EU799965.1.1501	unique	B30
p__Proteobacteria,_f__Rhodobacteraceae_FR683203.1.1468	unique	B33
p__Bacteroidetes,_f__Cryomorphaceae_FJ744882.1.1365	unique	B33
p__Proteobacteria,_f__Thiotrichaceae_AM176844.1.1462	unique	B33
p__Proteobacteria,_c__Alphaproteobacteria_EU802598.1.1454	unique	B33
p__Proteobacteria,_f__OM60_EU672862.1.1492	unique	B33
p__Proteobacteria,_f__Helicobacteraceae_DQ415790.1.1470	unique	B33
p__Proteobacteria,_f__Helicobacteraceae_DQ395005.1.1480	unique	B33
p__Proteobacteria,_f__HTCC2188_FR684306.1.1501	unique	B33
p__Proteobacteria,_f__Hyphomonadaceae_EU642858.1.1371	unique	B33
p__Proteobacteria,_f__Helicobacteraceae_GQ261309.1.1421	unique	B33
p__Proteobacteria,_f__Methylophilaceae_EU236430.1.1397	unique	B33
p__Bacteroidetes,_f__Cryomorphaceae_FR684954.1.1497	unique	B33
p__Proteobacteria,_f__Rhodobacteraceae_New.ReferenceOTU1016	unique	B33
p__Proteobacteria,_f__Rhodobacteraceae_DQ223020.1.1315	unique	B33
p__Proteobacteria,_f__SUP05_New.ReferenceOTU264	unique	B33

p__Proteobacteria,_f__OM60_AACY020294209.1241.2637	unique	B33
p__Proteobacteria,_c__Alphaproteobacteria_EU802596.1.1445	unique	B35
p__Proteobacteria,_f__Halomonadaceae_FJ745129.1.1300	unique	B35
p__Bacteroidetes,_f__Flavobacteriaceae_JQ194928.1.1360	unique	B35
p__Bacteroidetes,_f__Flavobacteriaceae_KC250891.1.1313	unique	B35
p__Bacteroidetes,_f__Flavobacteriaceae_JX524927.1.1427	unique	B35
p__Proteobacteria,_f__Alcanivoracaceae_DQ270698.1.1405	unique	B35
p__Proteobacteria,_c__Alphaproteobacteria_JQ196221.1.1307	unique	B35
p__Proteobacteria,_c__Alphaproteobacteria_New.ReferenceOTU601	unique	B35
p__Bacteroidetes,_o__Flavobacteriales_HQ242587.1.1485	unique	B35
p__Proteobacteria,o__Oceanospirillales_GQ348037.1.1385	unique	B35
p__Proteobacteria,_c__Alphaproteobacteria_EU799386.1.1375	unique	B35
p__Proteobacteria,_f__Rhodobacteraceae_GU235798.1.1298	unique	B35
p__Proteobacteria,_f__Rhodobacteraceae_New.ReferenceOTU610	unique	B35
p__Proteobacteria,_c__Alphaproteobacteria_KC425564.1.1450	unique	B35
p__Proteobacteria,_f__Pelagibacteraceae_EF572256.1.1435	unique	B35
p__Proteobacteria,_f__OM60_AY663906.1.1228	unique	B35
p__Proteobacteria,_f__Rhodobacteraceae_DQ421674.1.1299	unique	B35
p__Bacteroidetes,_f__Flavobacteriaceae_EU801694.1.1511	unique	B35
p__Verrucomicrobia,_f__Verrucomicrobiaceae_CAGN02000147	unique	B35
p__Cyanobacteria,_f__Synechococcaceae_HQ672155.1.1438	unique	B35
p__Proteobacteria,_f__Pelagibacteraceae_EU802434.1.1365	unique	B35
p__Proteobacteria,_f__Halomonadaceae_EU259792.1.1496	unique	B35
p__Bacteroidetes,_f__Flavobacteriaceae_GU940946.1.1406	unique	B35
p__Bacteroidetes,_f__Flavobacteriaceae_JQ197126.1.1360	unique	B35
p__Bacteroidetes,_f__Flavobacteriaceae_JQ200226.1.1360	unique	B35

Supplementary 5: Bacterial families significantly correlated with salinity. The top 5 most significant bacterial families showing either negative or positive Spearman correlations to salinity (bonferroni p value <0.01).

Bacteria fam.	**Spearman's statistics**	**P values**
Comamonadaceae	-0.8425	6.27E-52
Oxalobacteraceae	-0.8328	2.62E-49
Rhodocyclaceae	-0.8191	6.03E-46
Chitinophagaceae	-0.7865	3.47E-39
Cytophagaceae	-0.7449	2.01E-32
Halomonadaceae	0.8566	0
Piscirickettsiaceae	0.8274	0
Pelagibacteraceae	0.806	0
Vibrionaceae	0.7961	0
Synechococcaceae	0.7268	0

Supplementary 6: Spearman's correlation values between environmental variables and bacterial community. Spearman's values for temperature, salinity, precipitation and dissolved oxygen (DO), for each water mass.

Water mass	temperature	salinity	precipitation	DO
IS	0.6744	0.0555	0.2078	-0.0778
MS	0.5931	0.4872	0.2843	0.4413
OS	0.5762	0.642	0.3633	0.0545
B30	0.4548	-0.0699	0.1564	0.3371
B33	0.2158	0.0279	0.1569	0.1267
B35	0.3039	0.2318	0.4578	0.3476

Supplementary 7: Bacterial families significantly correlated with temperature. The top 5 most significant bacterial families showing negative or positive Spearman correlations with temperature (bonferroni p value <0.01).

Bacteria fam.	Spearman's statistics	P values
Pseudomonadaceae	-0.6482	1.70E-21
Sphingobacteriaceae	-0.6465	2.40E-21
Holophagaceae	-0.6214	3.01E-19
Lachnospiraceae	-0.6163	7.54E-19
Ruminococcaceae	-0.5909	5.59E-17
Verrucomicrobiaceae	0.6341	0
Microbacteriaceae	0.5789	4.44E-16
Cryomorphaceae	0.5786	4.44E-16
Balneolaceae	0.5671	2.22E-15
Erythrobacteraceae	0.5281	4.33E-13

Supplementary 8: Unique OTUs in Galindo tributary. OTUs that were significantly more abundant in Galindo river (Kruskal-Wallis, FDR value <0.01) and that were not present in the rest of estuaries/tributaries (and thus were unique for Galindo tributary) are shown in the table.

OTU	Average	Taxonomy
OTU1	1.7991	k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__Sphingobacteriaceae
OTU2	1.6197	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae
OTU3	1.4525	k__Bacteria;p__OD1;c__ZB2
OTU4	1.4004	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria
OTU5	1.3498	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria
OTU6	1.342	Unassigned
OTU7	1.3302	k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales
OTU8	1.3056	k__Bacteria;p__OP3;c__koll11;o__GIF10;f__kpj58rc
OTU9	1.2800	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__MIZ46
OTU10	1.2323	k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Synergistaceae
OTU11	1.2143	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria
OTU12	1.2023	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__MIZ46
OTU13	1.1348	k__Bacteria;p__OD1;c__ABY1
OTU14	1.1306	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria
OTU15	1.1034	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__BD7-3
OTU16	1.0941	Unassigned
OTU17	1.0525	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibrionaceae;g__Bdellovibrio
OTU18	1.0228	Unassigned
OTU19	1.0217	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria
OTU20	1.0138	Unassigned
OTU21	0.9565	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Oscillospira
OTU22	0.9298	k__Bacteria;p__Chlamydiae;c__Chlamydiia;o__Chlamydiales;f__Criblamydiaceae
OTU23	0.9225	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibrionaceae;g__Bdellovibrio
OTU24	0.8696	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Chromatiales;f__
OTU25	0.8688	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibrionaceae;g__Bdellovibrio
OTU26	0.8451	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__
OTU27	0.8447	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Bdellovibrionales;f__Bdellovibrionaceae;g__Bdellovibrio
OTU28	0.8395	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Spirobacillales
OTU29	0.8212	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae
OTU30	0.8169	Unassigned
OTU31	0.8113	k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__Cystobacterineae
OTU32	0.8043	k__Bacteria;p__Cyanobacteria;c__4C0d-2;o__MLE1-12
OTU33	0.7786	k__Bacteria;p__Proteobacteria;c__TA18;o__CV90
OTU34	0.777	k__Bacteria;p__Lentisphaerae;c__[Lentisphaeria];o__Victivallales;f__Victivallaceae
OTU35	0.7708	k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__HA64
OTU36	0.7608	k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Saprospiraceae
OTU37	0.6795	k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Rhodocyclales;f__Rhodocyclaceae;g__Zoogloea
OTU38	0.6741	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales
OTU39	0.6566	k__Bacteria;p__OP3;c__koll11;o__GIF10
OTU40	0.6515	k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales
OTU41	0.5548	k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Legionellales;f__Coxiellaceae
OTU42	0.5453	k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae
OTU43	0.5062	k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Helicobacteraceae;g__Sulfurimonas

Chapter 3:

Bacterial metabolic changes along an anthropogenically impacted European estuary

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Abstract

Estuaries are characterized by a marked physicochemical gradient between the freshwater of the river to the oceanic saline water. This environmental variations cause alterations in the microbial activity and composition/structure. Thus, studies along the salinity gradient of this ecosystem allow characterizing the intensity of those metabolic and community changes. Being metagenomic and metatranscriptomic ideal approaches to unravel the complex relationship between microbes, their function and their response to environmental changes, in this study the bacterial community of the Estuary of Bilbao was analyzed through both techniques in parallel. Thereby an in depth understanding of the inactive and active bacteria, and the latters metabolism changes along the estuarine gradient was obtained. For instance, the study sheds light on the bacterial metabolic changes occurring when they pass from oxygenated waters to stagnant-anoxic waters, where bacteria responds with the over-expression of anaplerotic and anaerobic metabolic pathways. Additionally, the abundance and the metabolic activity of the most abundant bacterial family described in this habitat, *Comamonadaceae* family, was characterized. The whole genome of two strains of *Limnohabitans* genus were recruited, and the gene transcription abundances changes of these bacteria were measured along the different water masses of the estuary identifying the differential expression of diverse functional pathways. For instance, the activation of cellular movement and membrane transport proteins were evidenced in stagnant eutrophic waters for *Limnohabitans* bacteria. Finally, the Estuary of Bilbao has been under great anthropogenic impact since 19th century, coming from industry, Waste Water Treatment Plant (WWTP) and sewage discharges which was reflected in the overall bacterial community found: e.g. *Rhodoferax*, *Chloroflexus*, *Bdellovibrio* or *Nitrospira*.

Introduction

Estuaries are one of the most largely anthropologically impacted coastal ecosystem and one of the most dynamic, complex and species rich habitat [1]. In this ecosystem the natural gradients of physicochemical conditions, from the river to the mouth of the estuary, causes heterogeneity in activity and composition of the microbiota [2,3]. Thus the studies of bacterioplankton communities in anthropogenically impacted estuaries are important to understanding the interaction of microorganism with the different environmental alterations that occur along these ecosystems [4]. In this way, the metabolic changes and adaptation of the bacterial communities to the different pressures could be describe [5].

The Estuary of Bilbao, the case study estuary of this work, has suffered structural modifications, whereby the large-scale reclamation of intertidal areas reduced the original estuary to a simple tidal channel in the mid-19th century [6]. This channelization changed the water circulation and turnover patterns, which modified both the abiotic and biotic processes and seasonal patterns in the plankton community [7]. In addition, since the beginning of the industrial age, wastes coming from the city or the factories have modified this ecosystem [8]. Currently the estuary is undergoing a recovery program since 1992 [8,9], but despite the improvement in environmental quality due to replacement of most polluting industries and the progressive implementation of an integrated sewage treatment plant, chronic pollution by metal and hydrocarbons still remains in sediments and water [10,11]. In addition, low Dissolved Oxygen (DO%) concentrations have been identified in the estuary caused by pollutants from emerging sources contaminant such as domestic sewage, organic compounds or nutrients [9]. Thus, to reduce the domestic sewage of the Bilbao metropolitan area, a Waste Water Treatment Plant (WWTP) was constructed in the shore of Galindo river, a tributary of the Estuary of Bilbao.

The bacterial community of this estuary has been previously studied by Aguirre and colleagues 2017 [12] by means of 16S rDNA gene amplicon sequencing method. Such technique allowed reaching to the taxonomic and the spatio-temporal analysis of the un-culturable bacterial community in this ecosystem. In the principal tributary of this estuary, Nervion-Ibaizabal river, Aguirre and colleagues (2017) evidenced members of Comamonadaceae family to be the most abundant bacteria, no matter the season [12], and its abundance were among the highest detected compared to other studied estuaries [13–15]. Interestingly, a gradient of its relative abundance was observed, decreasing as the freshwater of Nervion-Ibaizabal river was diluted in the oceanic saline water. In the same study the effect of the effluent water of the WWTP in the Galindo river [12] with the detection of typical bacteria of activated sludge tanks of the water treatment (such as *Bdellovibrio* and *Zoogloea* genus bacteria) [16,17]. While this previous work allowed describing the community composition, making assumptions on the functional potential of this community would be biased as it is based on one gene solely. In order to obtain an unbiased insight into community metabolic potential, sequencing the total community DNA rather and specific gene (16S rDNA gene), so called shotgun metagenomics, is considered to be a more appropriate approach. Shotgun metagenomic will allow the detection of the genomes and potential genes of the microbial community existing in the samples [18]. However, amplicon and shotgun metagenomics, are both DNA based approaches that do not distinguish among living and no-living (non active) microorganism, and thus, this limitation can cause erroneous conclusion of the functioning of the ecosystem [19], especially in ecosystems characterized by high physico-chemical changes. On the contrary, Metatranscriptomic analysis detects living and active microorganisms and their metabolism thus allowing the detection of the bacterial genes that are active [20], (but we can not detect the enzymatic activity of the proteins encoded in these genes).

In the present study we apply a combined metagenomic and metatranscriptomic approach to samples from the estuary of Bilbao and its main tributaries, simultaneously investigating the taxonomic composition, gene content and gene expression patterns of the bacterial community in

this ecosystem. We aimed to reveal the overall functional activities of this community and the specific gene transcriptional behaviors of organisms such as *Comamonadaceae* family, the most abundant organisms in this estuary according to a previous 16S amplicon study [12], in response to the salinity gradient.

Material and methods

Study area

The Estuary of Bilbao -the last track of the Nervion-Ibaizabal River- is a small macro-mesotidal system located on the Basque coast, north of the Iberian Peninsula, on the Cantabrian Sea coast (43º19'N 3º1'W). The Estuary of Bilbao crosses Bilbao's metropolitan zone (~1 million inhabitants) which is the center of the Biscay industrial area. The estuary is a 20 kilometers long narrow (50–2980 m wide) and shallow (6–30 m deep) channel. It was one of the most contaminated regions in Europe until the late 1980's and has since undergone a water recovery program [9]. Except for short periods of high river discharge, euhaline waters (salinity >30 ppt) dominate within the estuary [21]. The Estuary of Bilbao is partially mixed in the outer portions and highly stratified within the inner half; in the upper layers we found freshwater while the bottom layers remain euhaline [22]. This stratification is given in such a way that freshwater is located in the upper layers while all the bottom remains euhaline. This stratification is a consequence of channeling the original estuary [6], which caused the freshwater to begin flowing solely through the upper stratum bypassing the bottom saline water.

The Estuary of Bilbao has ample tributaries, with several differences in their water input: Nervion-Ibaizabal (66% of the water input of the Estuary of Bilbao), Kadagua (27%), Asua (0.7%), Galindo (4%) and Gobela (0.3%) [23]. These tributaries flow through different areas with different environmental characteristics. Among them, Nervion-Ibaizabal and Galindo rivers have been studied. On the one hand, Nervion-Ibaizabal river is the biggest tributary, and it is characterized by a variable river discharge along the different seasons (4.5-37 $m^3 s^{-1}$) and by its basin of 1.900 Km^2, which is the most extensive of the Biscay. On the other hand, Galindo river is under the influence of the discharges of the Waste Water Treatment Plant (WWTP) and the metallurgical industry that

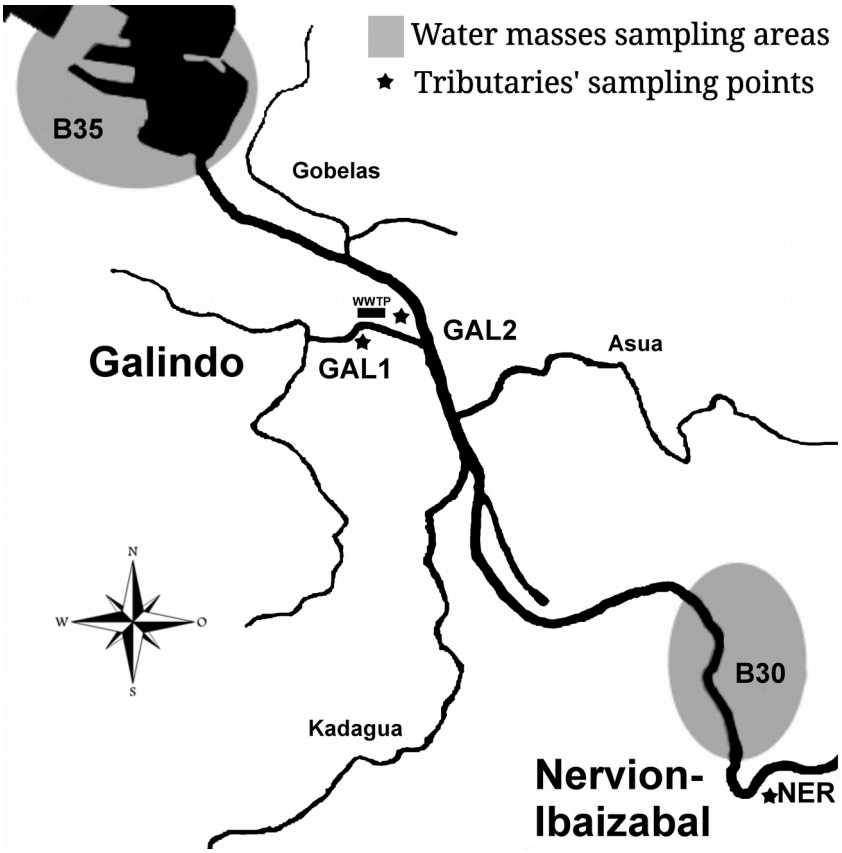
was located on the river since XIX century and, consequently, in the river sediments different industrial pollutants were deposited [6]. The WWTP has an average daily effluent flow of 4,000 l / sec (according to the data provided by the WWTP, http://www.bizkaia21.eus/atalak/TerritorioSostenible/Lugares/datos.asp?id=3&IdPagina=36&idioma=ca). The Galindo river is a low flowing river, its discharge never reaches more than 3,000 l / sec (except in large rainfalls, [7]). In summer its discharge barely reaches 500 l / sec. For this reason, most of the Galindo river water has its origin in the WWTP.

Sample collection and physical and chemical analyses

Sampling was conducted within the tributaries of Nervion-Ibaizabal (NER) and Galindo (GAL1 and GAL2) on the 7th of August in 2014. Samples were collected in the last stretch of each tributary and from fixed points (bridges mostly), avoiding areas affected by the tide. In the case of Galindo, the GAL1 sampling station was just 5 meters from the outlet of the WWTP, and GAL2 sampling station was in the last tract of the Galindo river, which is under the influence of saline water.

The sample collection within the estuary was carried out on 5th August 2014. Sampling took place in the days of neap tide coefficient (30-50), at high tide and at about the same moment of the day of the tributaries sampling (10 to 12 am), to eliminate confounding variables. Estuarine samples were collected in two salinity water masses: 30 and 35 ppt salinities, herein B30 and B35 respectively (Fig. 1).

Figure 1: Sampling stations at the Estuary of Bilbao and its tributaries. Highlighted in gray are the areas where the salinity water masses of 30 and 35 ppt were located, where B30 and B35 samples were sampled. The stars indicate the sampling points within tributaries, in this study NER, GAL1 and GAL2. WWTP refers to the Waste Water Treatment Plant location.



The 5 samples analyzed in this study; NER, GAL1, GAL2, B30 and B35; were collected using an oceanographic Niskin bottle. For each of these samples metatranscriptomic and metagenomic approaches were carried out in parallel. Samples for metatranscriptomic analyses were filtered *in situ*, using 0.22 µm Durapore® membrane filters (Millipore, 25 mm diameter) in a portable holder and a syringe. After the filtration, the filters were store in liquid nitrogen, until RNA extraction. Samples for shotgun metagenomic analysis were collected at same point and time. Those samples were stored in opaque plastic jerry cans in the field. Once in the laboratory, the water was filtered (5 L approx.) through 20 µm Nylon net filters (Millipore, 90 mm diameter) and bacteria were collected with 0.22 µm Durapore® membrane filters (Millipore, 47 mm diameter). Filtration was

performed in triplicate using a Kitasato Flask and a vacuum pump. The whole process, from sampling to storage, took less than 3 hours to perform. All filters were store at -80 ºC until DNA extraction.

At each sampling point, measures of salinity, temperature, pH, and dissolved oxygen saturation (DO%) were obtained in situ using a YSI 556 MPS Multiparameter Probe. Water transparency was measured with a Secchi Disk. Chlorophyll concentrations were calculated from spectrophotometric measurements on acetone extracts using a monochromatic method with acidification [24]. The physico-chemical characterization of the 5 samples collected -two riverine samples (GAL1 and NER) and three estuarine samples (GAL2, B30 and B35)- is summarized in table 1.

Sample	Secchi depth (m)	Salinity (ppt)	Temperature (ºC)	DO (%)	pH
NER	1.5 (bottom)	0.42	21.31	98.1	8.35
GAL1	2 (bottom)	1.25	24.2	82.5	8.06
GAL2	2.5	28.67	22.9	59.6	7.97
B30	0.75	29.54	22.37	8.3	7.72
B35	5.5	34.4	22.04	107.9	8.11

Table 1: Environmental variables information of each sample.

DNA extraction and sequencing for Shotgun metagenomics

Total genomic DNA was extracted from the half of the 0.22 µm Durapore® membrane filters using PowerSoil DNA isolation kit (Mo Bio laboratories, Inc., Carlsbad, CA, USA) following the manufacturer protocol. The DNA quantity and quality of each sample was assessed by either a ND-1000 spectrophotometer (NanoDrop) or Qubit fluorimeter (Life technologies). Finally, the DNA extractions were stored at -20 ºC until DNA sequencing.

The sequencing of the metagenomes was carried out on a Illumina's HiSeq 2000 system, applying the Paired-End DNA Sample Preparation Kit v4 (Illumina Inc.) as described by the manufacturer to generate 2 × 125 bp paired- end reads in the "Centro de Análisis Genómico (CNAG)", Spain.

RNA extraction and sequencing for metatranscriptomic

Total microbial RNA was extracted from the half of the 0.22 μm Durapore® membrane filters using Trizol (Invitrogen) RNA extraction method. Next, RNA purification was performed using PureLink RNA Mini kit (AMBION) combining with PureLink Dnase (AMBION) to carried out a DNAse treatment. Then ribosomal RNA (rRNA) depletion was performed using Ribo-Zero Magnetic kit for Bacteria (Epicentre) and the re-purification of microbial mRNA was achieved by MICROBExpress kit (AMBION). Finally, the messenger RNA (mRNA) quality was measured by Bioanalizer 2100, using RNA 6000 Nano chip. Finally, the RNA extracts were stored at -80 ºC until sequencing.

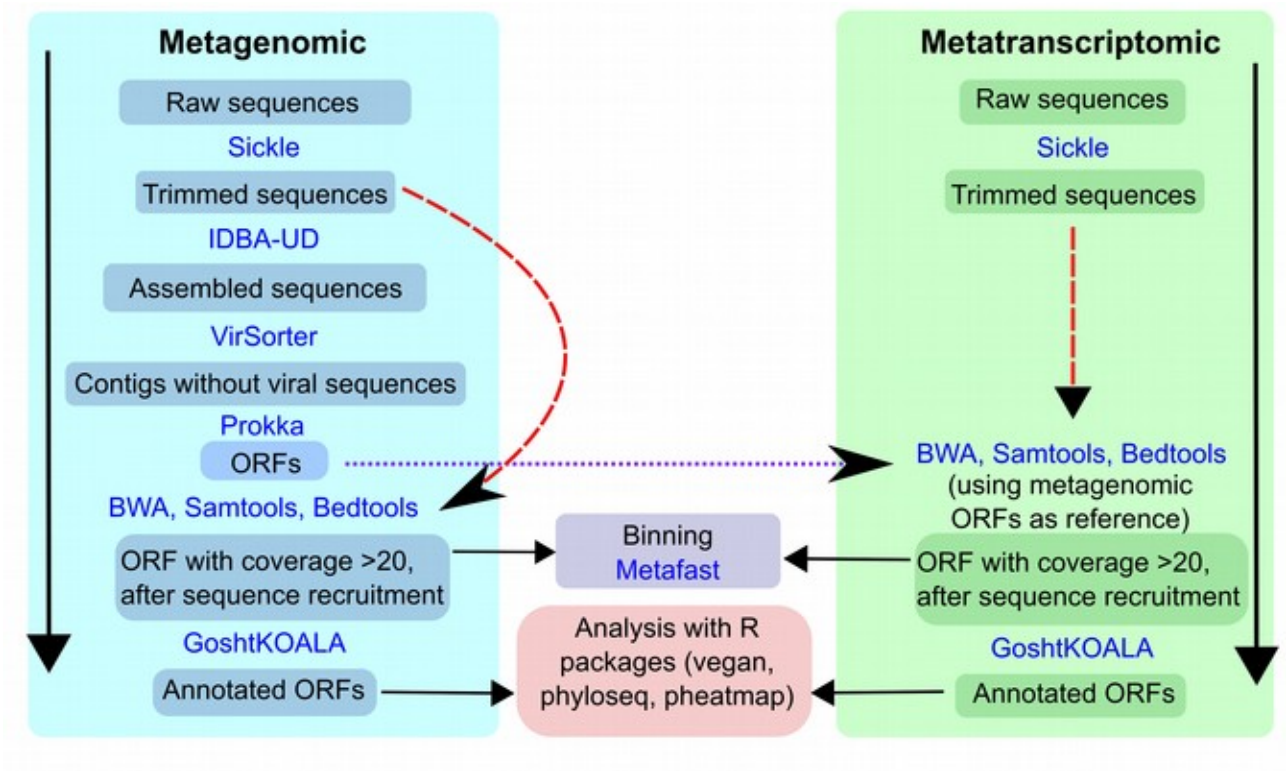
The sequencing of the metatranscriptomes was carried out on a Illumina's HiSeq 2000 system, applying the Stranded mRNA-Seq library preparation kit (Illumina Inc.) as described by the manufacturer to generate 2 × 100 bp paired- end reads by the "Centro de Análisis Genómico (CNAG), Spain.

Bioinformatic pipeline

Metagenomic data was first trimmed using Sickle tool (v1.33) [25] with default parameters (including Phred score ≥ 20, Fig. 2). Trimmed sequences were assembled using idba-ud [26] version 1.1.1, testing a range of k-mer sizes from 20 to 100 in steps of 20. Kmer size 100 yielded the most contiguous assembly result and was chosen for subsequent analysis. In the assembled dataset viral sequences were identified using VirSorter 1.0.3 software [27], and these sequences

were removed by filter_fasta.py command in QIIME software (v1.9.1) [28]. After virus sequences removal, an Open Reading Frame (ORF) search was carried out using PROKKA software (v.1.11) [29] and the sequences that were classified as transporter RNA (tRNA) and ribosomal RNA (rRNA) were removed using Aragorn [30] and RNAmmer [31] algorithms respectively.

Figure 2: Flow diagram of the bioinformatics analysis pipeline for metagenomic and metatranscriptomic sequences. The software used are in blue. The red arrows with intermittent lines indicate the sequence recruitment (trimmed sequences against the identified ORFs) carried out by BWA software. The arrow with purple dots indicates the usage of metagenomic ORFs as reference for metatranscriptomic sequences to identify active genes.



Trimmed sequences were aligned to the assembled ORFs using BWA software [32] for estimating the inclusivity of the ORFs (Fig. 2). Then SAMtools version 1.1 [33] was used to convert SAM files to BAM, sort the alignment file and calculate the mapping statistics. The coverage of each ORF was calculated using BEDtools (v2.17.0) [34] and a home-made script in R

(https://www.protocols.io/view/metagenomic-analysis-huib6ue). The ORFs with Q>30 and a mean coverage greater than 20 were selected.

To identify which of the genes were actually active in the community, the ORFs obtained from metagenomic sequences were used as a reference to map metatranscriptomic reads (previously trimmed with sickle software [25]) using BWA [32]. Then SAMtools [33] was used to calculate the mapping statistics selecting the ORFs with Q>30 and mean coverage>20 (Fig. 2).

Total metagenome's ORFs and active ORFs datasets were then used for their taxonomic (genus level) and functional assignment (KEGG Orthology level) against KEGG database [35] using GhostKOALA [36]. Subsequently, by a home-made script in R [37] a heatmap of the presence of genus and functions was carried out, by water mass and dataset (metagenomes ORFs and metatranscriptomes ORFs), using the package Pheatmap [38]. In this way, we compared the euclidean distance differences between potential taxa/functional abundances (metagenomics) vs the active taxa/functional abundances (metatranscriptomics). Moreover, the community/functional dissimilarity within the different sample-points of the estuary and its tributaries was determined by Canonical Correspondence Analysis (CCA) in biplot format. This analysis was carried out by phyloseq (v. 1.14) R package [39] (Fig. 2). Finally, in order to detect which KEGG modules were more active in each sample, the functions that were expressed within the Q1 (the functions that their relative abundances were above of the 75 percentile) of each sample were selected. The relative abundances were calculated by the formula: 100 x (average coverage of the ORF) / (Total average coverage of the ORFs). Subsequently, highlighted modules of each sample were compared with the others through an analysis of Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/). In this way, the functional modules that were shared among the samples and the characteristic modules of each sample will showed.

Additionally, the binning of the reads was carried out by Metafast software (v 0.1.1) [40] from metagenomes ORFs and active ORFs datasets. Bray-Curtis distance matrices were calculated from both datasets to construct heatmaps and dendrograms to show differences among the bacterial communities of the different samples (Fig. 2).

To analyze the genome recuperation/inclusivity of Comamonadaceae (previously shown to be as the most abundant bacterial family in this estuary [12]), the reads obtained from the metagenomic and metatranscriptomic analysis were mapped against all publicly available reference genomes of members of this family (obtained from NCBI database), using BWA [32]. Then SAMtools [33] was used to convert SAM to BAM and sort the alignment file. Next, the coverage of each reference genome was calculated using BEDtools (v2.17.0) [34]. The regions with higher coverage than 3 were taken into account for this analyzes. Finally, the percentage of recovered genomes for each Comamonadaceae family member was calculated using a home-made R script (https://www.protocols.io/view/metagenomic-analysis-huib6ue).

Results

Our sequencing effort yielded 285.9 Gbp in total for metagenomic sequencing and 49.615 Gbp for metatranscriptomic sequencing. After the bioinformatic pre-processing (trimming, assembly, viral sequencing depletion, ORF finding, reads-recruitment) we detected an average of 354.732 ORFs per sample for metagenomic analysis. The 35.36% of the metagenomic trimmed reads were recruited into these identified ORFs. For the metatranscriptomic analysis, 28,9 % of the trimmed transcripts were mapped against the ORFs (constructed from metagenomic data) (Table 2).

A) Metagenomic samples's information

Sample Name	Yield (Gb)	Million reads	trimmed reads % (Q=20 & l=100)	Metagenomic reads mapped against metagenomic ORFs (%)	Metagenomic ORFs
NER	56833	451,05	91,96	34,37	366985
GAL1	59309	470,71	90,35	40,89	383195
GAL2	56928	451,8	91,71	42,24	331120
N30	56587	449,1	92,21	39,22	346971
N35	56288	446,73	91,55	35,09	345391
Total	285945	2269,39	NA	NA	NA
Average	57189	453,88	91,55	38,36	354732,4

B) Metatranscriptomic sample's information

Sample Name	Yield (Gb)	Million reads	trimmed reads % (Q=20 & l=100)	Metatranscriptomic reads mapped against metagenomic ORFs (%)	Metatranscriptomic ORFs	Percentaje of metatranscriptomic ORFs contrasting metagenomic ORFs
NER	13,34	132,12	77,96	22,67	34097	9,29
GAL1	7,54	74,65	90,91	21,59	20128	5,25
GAL2	12,11	119,86	75,61	33,81	30302	9,15
N30	9,87	97,72	89,93	35,26	44734	12,89
N35	6,76	66,9	84,27	31,14	23834	6,90
Total	49,62	491,25	NA	NA	NA	NA
Average	9,92	98,25	83,74	28,9	30619	8,70

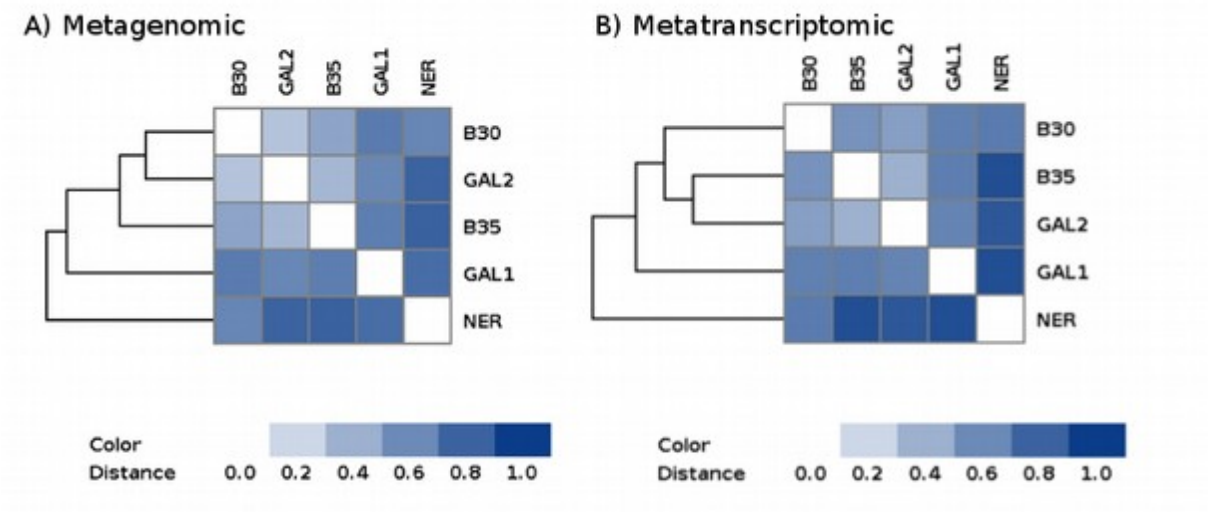
Table 2: Sequencing yield and processing statistics. A) information of metagenomic samples. B) Information of metatranscriptomic samples.

Binning results

The dissimilarity distances obtained from the binning analysis (Fig. 3) of metagenomes and metatranscriptomes datasets showed that saline water samples (B30, B35 and GAL2) grouped together, apart from the freshwater samples (NER and GAL1). The Bray-Curtis (BC) distances between the samples analyzed by metatranscriptomics were higher (BC <0.907) than for metagenomic analysis (BC <0.808). Overall, for both datasets, Nervion-Ibaizabal samples (NER) showed the highest dissimilarity distances from the rest of samples (BC >0.621), while the saline samples (B30, B35, GAL2) had lowest Bray-Curtis values between them (BC <0.555).

Figure 3: Heatmap of estuarine and tributarine samples for metagenomic (a) and metatranscriptomic (b) approach.

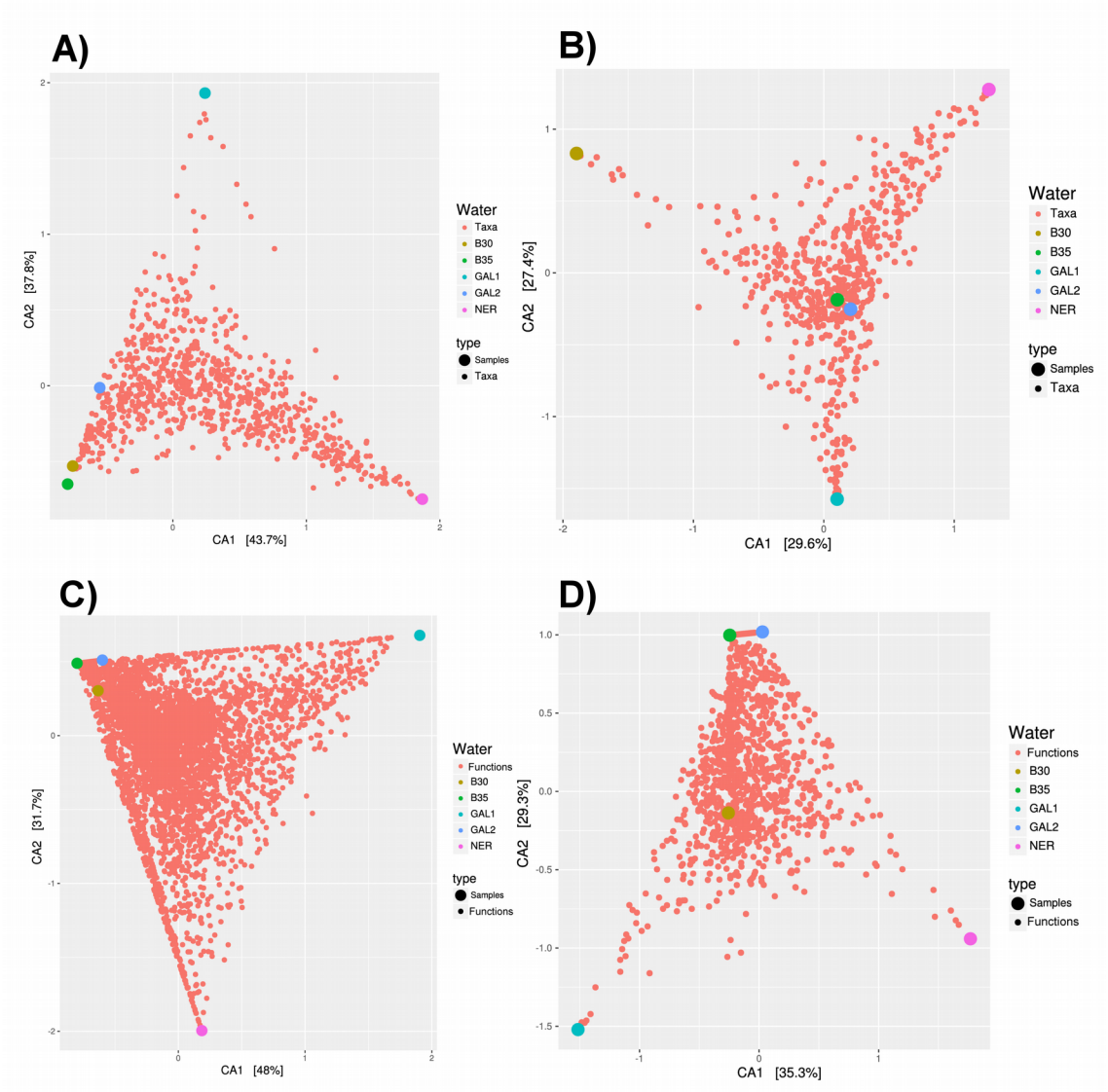
Dendrogram sample classification based on distance matrix of k-mers of the sequences of each sample, constructed using Metafast software. The distances were calculated based on the k-mer dissimilarity distances and represented in Bray-Curtis distances (BC).



Taxonomic and functional analysis of metagenomes and metatranscriptomes

CCA ordination plots and heatmaps created from the Taxonomic and functional annotation of both metagenomes and metatranscriptome ORF datasets (GhostKOALA software output) showed a similar sample distribution (Fig. 4, Supplementary 1, Supplementary 2) than the once obtained in the binned results (Fig. 3): 1) GAL1 and NER samples, were always distanced from the saline water samples and, between them, they had a differentiated community and functional profiling (Fig. 4, Supplementary 1, Supplementary 2). Within saline water samples (GAL2, B35, B30), metagenomic data analysis resulted in a similar community and potential functional composition (Fig. 4A and 4C, Supplementary 1, Supplementary 2). 3) On the contrary, when studying the metatranscriptomic dataset, B30 sample community and active functional profiling was highly differentiated from the rest of saline water samples (GAL2, B35) (Fig. 4B and 4D, Supplementary 1, Supplementary 2).

Figure 4: CCA biplots of annotated taxonomic and functional abundances for metagenomic and metatranscriptomic data. Taxonomic and functional distribution for metagenomic and metatranscriptomic analysis of the results of GhostKOALA. A) Taxonomic distribution for metagenomic data. B) Taxonomic distribution for metatranscriptomic data. C) Functional distribution for metagenomic data. D) Functional distribution for metatranscriptomic data.



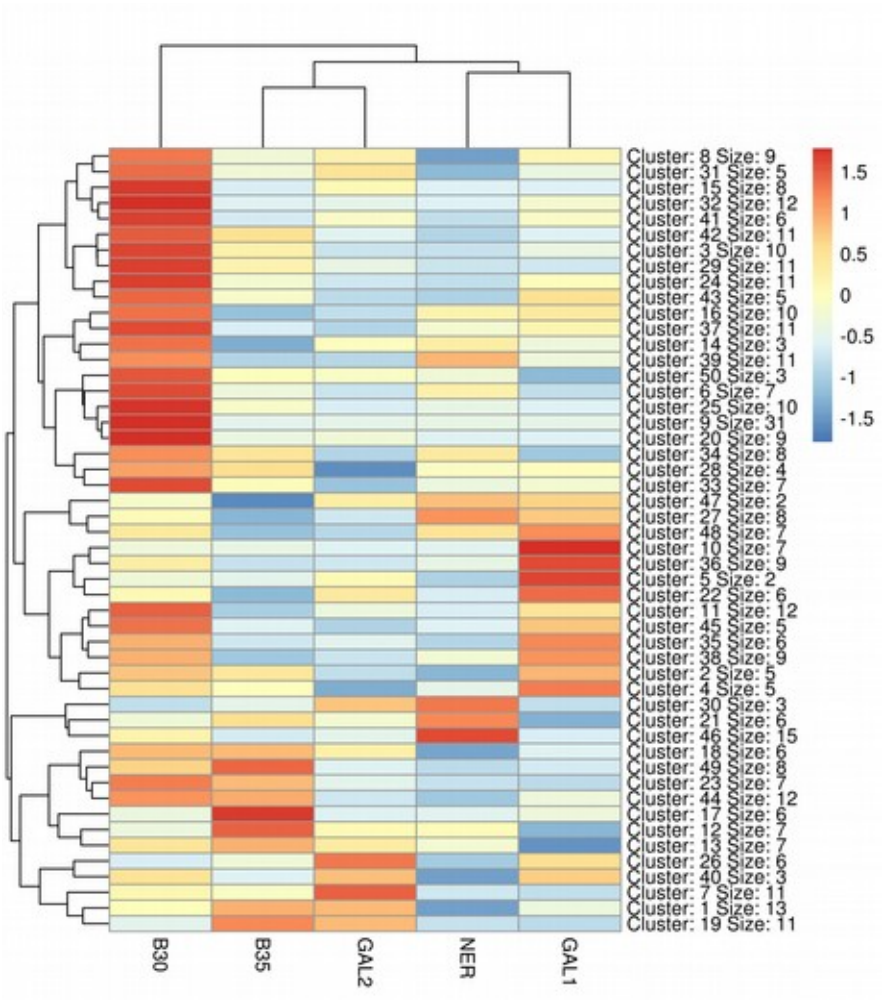
It was noted that each river (NER and GAL1) had a specific taxonomic cluster (Supplementary 1) composed by higher abundances of *Limnohabitans* (NER = 6.5%, GAL1 = 2.1%), *Flavobacterium*

(NER= 3%, GAL1=2.6%), *Fluviicola* (NER=2.2%, GAL1=1.2%), etc, which their abundance decreasing as salinity increased. Among them Nervion-Ibaizabal river had greater taxonomic input into the estuary as; the most abundant bacterial taxa of this river (e.g (*Limnohabitans* (6.5%), *Flavobacterium* (3%), *Fluviicola* (2.2%), *Rhodobacter* (1.2%), etc) were also detected in the rest euhaline waters of the estuary. While many of the genus stem from NER practically disappear in the rest of the estuary (for example: *Desulfuromonas*, *Leptotrix*, *Castellaniella*), a good part of them are present in the inner B30 saline water mass, such as: *Limnohabitans* (1.6% in B30), *Flavobacterium* (1.5%), *Fluviicola* (1.8%), *Niastella* (0.5%), etc. In B30 water mass Cyanobacteria (14.1% of metatranscriptomic reads of this sample) and *Firmicutes-Clostridia* (37.3%) were the most active bacteria detected. The freshwater sample of Galindo (GAL1), also showed its own taxa cluster (Fig. 4), characterized by higher abundances of many genus: *Mycobacterium* (25.6%), *Flavobacterium* (2.6%), *Gordonia* (0.5%), *Rhodococcus* (1.5%), *Nitrosomonas* (0.2%), *Rhodoferax* (0.2%), *Bdellovibrio* (0.2%), *Nitrospira* (0.2%), among others.

When the genes with greater transcriptional abundance (KO numbers of the first quartile) within the metatranscriptomic dataset were analyzed, several activities (KEGG modules) highlighted from the rest (Supplementary 3): 1) Most of the activities in all waters were related with photosynthesis, energy metabolism, gene transcription and other basic live functions. 2) In the Nervion sample, modules related with multidrug resistance (Mex type, GesABC and AcrAB-TolC/SmeDEF pumps) were highlighted. 3) The comammox function was active in the two stations of the Galindo river (GAL1 and GAL2). 4) The most active functions in GAL1 were related with enzymatic activities such as indolepyruvate ferredoxin oxidoreductase and acetolactate decarboxylase (Supplementary 4). 5) B30 water mass had the largest block of characteristic functions (Supplementary 2, Supplementary 3), that included ethylmalonyl pathway, reductive acetyl-CoA pathway and hydroxypropionate-hydroxybutylate cycle. Moreover, some of the functions of B30 were shared with the vicinity waters, such as isoprenoid biosynthesis (also present in NER) and dissimilatory and assimilatory sulfate reduction (also present in GAL2 and B35). Furthermore, most of the genes

that were transcribed in B30 (Fig. 5, Supplementary 5) were related to reductive citrate cycle (Arnon-Buchanan cycle), dissimilatory sulfate reduction, or dicarboxylate-hydroxybutyrate cycle (Supplementary 6). Additionally, there were various set of genes whose transcription was lower in B30 when compared to GAL1 and B35 (Fig. 5, Supplementary 5).

Figure 5. The most active metabolic functions of B30 and their differential activity in the other samples. Heatmap based on KO objects with abundances located in the first quartile of the metatranscriptomic data of B30 sample. The differential abundance distribution of those KO objects between all the water masses are shown.



Comamonadaceae recruitment

Sequence recruitment against the reference genomes of the family *Comamonadaceae* indicated that these bacteria had higher abundances in freshwaters (NER and GAL1) and in the inner saline water mass of the estuary (B30) than in the outer water mass (B35) (Table 3). Interestingly, samples of the inner of the estuary (B30 sample) were the once with highest genomic recruitment. Most of recruited sequences were assigned to the reference genomes NZ_CP011774.1 and NZ_CP011834.1, both corresponding to two different strains of *Limnohabitans* genus (Table 3). In both cases the percentage of genome recovered was up to 95.32% and their average coverage reached 376 in the case of NER sample (Table 3). Similarly, both *Limnohabitans* genus strains showed the “highest average coverage” and highest “percentage of recovered genome” among the transcript assigned to *Comamonadaceae* family members (Table 3). While these two strains had the highest read recruitment in NER metagenomic dataset, their read recruitment in metatranscriptomic dataset was higher in B30 than in NER.

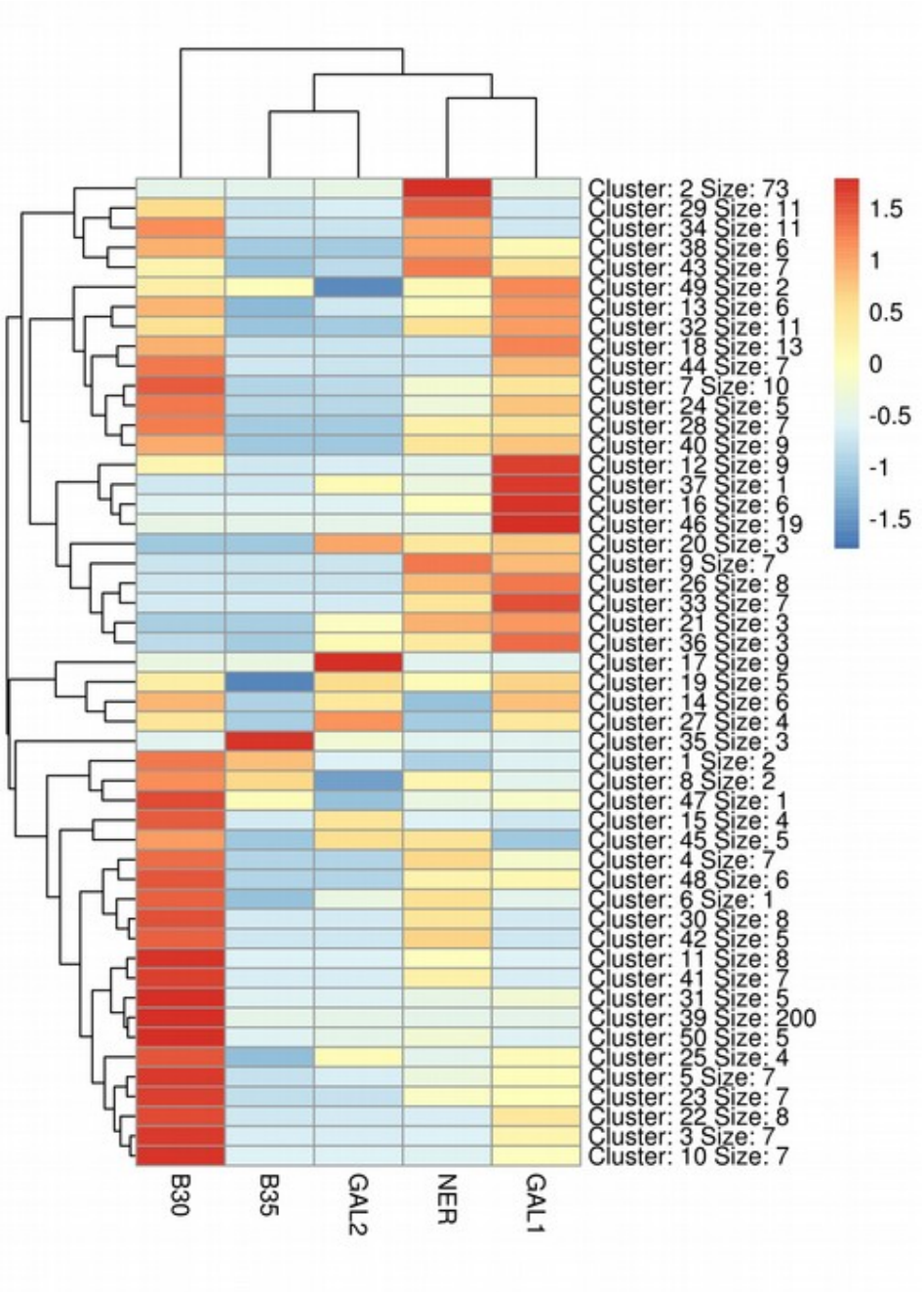
	Reference genome and taxonomy	Average coverage of genome					percentage of the recruited genome				
		B35	B30	NER	GAL1	GAL2	B35	B30	NER	GAL1	GAL2
Metagenomic	NZ_CP017311.1 Hydrogenophaga sp. PBC	0,48	2,55	7,33	4,71	2,93	2,43	16,79	26,14	24,80	8,42
	NZ_CP010951.1 Ramlibacter tataouinensis strain 5-10	0,50	1,94	4,08	4,08	1,96	2,27	12,14	19,36	21,50	6,19
	NZ_CP020121.1 Comamonas kerstersii	0,77	1,60	2,95	2,43	2,62	2,25	7,50	12,01	13,91	5,49
	NC_008782.1 Acidovorax sp. JS42	0,60	1,32	2,89	3,66	1,76	1,71	8,86	16,29	24,78	6,11
	NC_018708.1 Acidovorax sp. KKS102	0,28	1,86	5,18	5,67	1,81	1,58	13,44	20,88	45,33	7,34
	NC_015677.1 Ramlibacter tataouinensis TTB310	0,44	2,30	5,81	5,50	2,07	2,46	16,56	25,87	29,13	8,16
	NZ_CP021455.1 Comamonas serinivorans	0,68	1,53	3,06	2,51	1,84	2,11	8,10	12,64	13,32	4,91
	NZ_AP014568.1 Comamonadaceae bacterium A1	0,91	2,59	5,08	3,53	3,74	3,14	13,67	20,99	20,16	8,67
	NZ_AP014569.1 Comamonadaceae bacterium B1	1,10	2,86	5,36	4,08	4,36	3,40	13,94	20,82	21,31	9,25
	NZ_CP019239.1 Rhodoferax saidenbachensis	1,30	2,28	6,05	3,83	2,69	1,78	12,38	22,72	22,82	6,82
	NZ_CP011774.1 Limnohabitans sp. 63ED37-2	1,77	67,67	376,63	90,08	16,23	10,25	93,09	95,32	90,01	77,49
	NZ_CP011834.1 Limnohabitans sp. 103DPR2	1,34	43,52	151,10	25,56	8,95	6,39	71,07	76,77	66,29	42,54
	NZ_CP016447.1 Acidovorax sp. RAC01	0,34	2,01	5,57	5,51	1,99	1,90	15,32	22,33	33,18	8,18
	NZ_CP016449.1 Hydrogenophaga sp. RAC07	0,61	3,76	12,19	4,79	3,59	2,67	24,79	37,01	28,57	10,55
	NZ_CP015698.1 Curvibacter sp. AEP1-3	0,38	1,96	5,44	2,82	2,41	1,77	13,19	27,70	17,23	6,01
Metatranscriptomic	NZ_CP019240.1 Rhodoferax antarcticus	0,51	0,73	0,61	0,90	0,30	0,46	1,35	1,42	2,14	0,55
	NZ_CP016603.1 Comamonas aquatica strain CJG	0,32	0,41	0,37	0,53	0,20	0,46	1,29	1,43	2,42	0,56
	NZ_CP020121.1 Comamonas kerstersii strain 8943	0,55	0,60	0,47	0,71	0,28	0,71	1,46	1,61	2,54	0,78
	NC_018708.1 Acidovorax sp. KKS102	0,14	0,26	0,28	0,50	0,10	0,32	1,42	1,47	3,12	0,42
	NZ_CP006704.1 Comamonas testosteroni TK102	0,20	0,26	0,23	0,28	0,11	0,47	0,78	0,84	1,27	0,40
	NZ_AP014568.1 Comamonadaceae bacterium A1	0,24	0,41	0,42	0,46	0,16	0,57	1,59	1,72	2,20	0,77
	NZ_AP014569.1 Comamonadaceae bacterium B1	0,26	0,41	0,41	0,44	0,17	0,67	1,71	1,70	2,34	0,84
	NZ_CP019239.1 Rhodoferax saidenbachensis	0,52	0,80	0,81	1,08	0,33	0,43	1,88	2,37	3,22	0,68
	NZ_CP012073.1 Ottowia sp.	0,40	0,47	0,34	0,46	0,19	0,48	1,04	0,99	1,43	0,51
	NZ_CP021359.1 Acidovorax sp. NA2	0,25	0,37	0,36	0,58	0,15	0,38	1,11	1,32	2,50	0,45
	NZ_CP021366.1 Acidovorax sp. P4	0,25	0,36	0,36	0,58	0,15	0,37	1,13	1,33	2,49	0,43
	NZ_CP011774.1 Limnohabitans sp. 63ED37-2	0,77	7,86	14,02	8,91	0,85	2,00	37,37	26,23	23,44	3,70
	NZ_CP011834.1 Limnohabitans sp. 103DPR2	1,21	8,88	16,28	8,08	1,22	1,11	34,06	28,90	21,99	4,34
	NZ_CP017476.1 Hydrogenophaga sp. LPB0072	0,23	0,49	0,54	0,52	0,22	0,50	1,79	1,69	2,01	0,69
	NZ_CP016449.1 Hydrogenophaga sp. RAC07	0,20	0,49	0,56	0,55	0,18	0,48	2,46	2,36	2,70	0,63
	NZ_CP015698.1 Curvibacter sp. AEP1-3	0,11	0,31	0,51	0,67	0,17	0,49	1,87	2,50	2,83	0,70

Table 3: Comamonadaceae genomes coverage and sequence recruitment for metagenomic and metatranscriptomic analysis. "Average coverage of genome" column shows the highest average coverage (up to percentile 75 of the total) of genomes belonging to the *Comamonadaceae* family. For this analysis the regions with coverage equal or greater than 3 were taken into account.

The KO objects associated to the two strains of Limnohabitans in B30 belonged to reductive nitrate cycle (Arnon-Buchanan Cycle), Branched-chain amino acid transporters, Thiosulfate oxidation by SOX complex, the flagellar system or different secretion systems (Type I & II and Sec system) (Figure 6, see clusters KO classification in Supplementary 5). Whereas the KO objects associated to the two strains of *Limnohabitants* in riverine samples, NER and GAL1, showed high abundances

of F-type ATPase, pyrimidine biosynthesis and NADH: quinone oxidoreductase functions (Fig. 6, see clusters KO classification in Supplementary 5).

Figure 6: Limnohabitans metabolic activity among the different water samples. Heatmap plot of the KO objects related to Limnohabitans in the different samples of metatranscriptomic data. The classification of the KO objects associated to each cluster is given in Supplementary 5.



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Discussion

Estuaries are transition zones from freshwater to marine ecosystem, where ecological niches with particular physico-chemical characteristic and microbial communities occur [41]. Therefore, estuaries are interesting places to analyze the metabolic adaptation of bacteria to different environmental changes. Thus, in the present study we conducted a microbial research combining metagenomic and metatranscriptomic approaches to unravel the community composition and functional changes in the estuary of Bilbao. Five samples were analyzed from the main tributaries and the estuary: Two riverine-freshwater samples; Nervion-Ibaizabal (NER) and Galindo (GAL1), and three saline waters; GAL2 (the last track of Galindo river with salinity 28 ppt), B30 (inner estuary with salinity 30 ppt) and B35 (outer waters of the estuary with salinity 35 ppt). Similar to other studies, where the metagenomic and metatranscriptomic approaches have shown to be effective to identify potential and active function in various ecosystems (such as rivers, biogas plants and cow rumen [4,42,43]), in the present study the parallel use of those methodologies was able to unravel active bacterial metabolic pathways and how their transcription is modified along the estuary gradient (Fig. 4, Supplementary 5).

Within the samples studied, B30 water mass of the estuary of Bilbao had a particular/distinguishable active community and functional profile, differing from the rest of saline waters (GAL2 and B35) (Fig. 3, Fig. 4, Fig. 5, Supplementary 1, Supplementary 2, Supplementary 3). This water mass is characterized by high turbidity and low pH and DO concentrations leading other authors to classify this water mass as eutrophicated [7,9,22,44]. B30 sample had several functions that were significantly more expressed (Fig. 5, Supplementary 6), and many of them were indeed associated to eutrophication processes. For example, Ethylmalonyl pathway, a anaplerotic reaction to synthesize malate and succinate without oxygen was more abundantly expressed in B30 than in the rest of waters. A similar case was found for reductive acetyl-CoA

pathway, which has been associated to high CO^2 concentrations and methanogenic bacteria [45]. Accordingly, bacteria such as *Desulfobacterium* and *Planctomycetes*, which are known to use the reductive acetyl-CoA pathway for CO^2 fixation and energy conservation via generation of an electrochemical gradient in anaerobic medium [46], where shown to have highest abundances in NER and B30 samples (Supplementary 1). Likewise, the hydroxypropionate-hydroxybutylate cycle (Carbon fixation pathway) was identified to be more abundant in B30. This activity has been previously associated to *Chloroflexus* genus [47], and in accordance, in this study *Chloroflexus* showed highest abundances and activity in B30 and GAL1. Also, isoprenoid biosynthesis pathways (secondary metabolite of photosynthetic organisms) were more abundant in B30 and in NER samples than in the rest of samples (Supplementary 3). Holopainen and colleagues (2013) [48] described eutrophication of coastal areas to result in an increase of phytoplankton in aquatic ecosystems, which turned in a rapid increase of isoprene and monoterpenes emissions from algae and cyanobacteria [49–51]. In conclusion, all the later set of functions indicated that bacteria present in B30 water mass adapt their metabolism to survive to the anoxia and the high concentration of different compounds, such as chlorophyll or organic pollutants [9].

Regarding the taxonomic analysis, *Comamonadaceae* family was the most abundant bacterial family described in a previous work conducted in this estuary through 16S rDNA amplicon analysis [12]. In the present study Comamonadaceae family showed similar abundance patterns to the results found in the previous 16S rDNA amplicon study, where its abundance was highest in the Nervion-Ibaizabal (NER) sample and went decreasing with salinity. However the taxonomic analysis of the metagenomic and metatranscriptomic data were able to uncover the presence of different genus belonging to this bacterial family (Table 3). Between the different member of *Comamonadaceae* family, *Limnohabitans* was the most abundant genus in both metagenomic and metatranscriptomic dataset (Table 2). This genus has the capability of living in oxic and anoxic environments [52] predominantly of freshwater habitats, being sensitive to acidity and salinity. Therefore their occurrence in ocean or acidic environments is negligible. Also, this type of bacteria

grows using exudates produced by algae [53,54], and thus *Limnohabitans* is considered to be an opportunist fast-growing bacteria that takes advantage of phytoplankton blooms [55]. In accordance, the sample collected in Nervion-Ibaizabal river (NER) showed to have the highest abundance of this genus, and its affluence dwindled as it reached to oceanic waters. Interestingly, these bacteria changed their metabolism when they arrived to saline waters (30 ppt) (Fig. 6) where they activated functions related with the cellular movement and the membrane transport of many ions and peptides (Supplementary 5). These functions could be related with the drastic environmental changes associated to changing from freshwater to saline waters: 1) B30 water mass is more stagnant than the river water, which is always in movement. In this situation, cell-mobility function to be able to acquire food or to find an ecological niche more adapted to its functions becomes necessary [56]. 2) Within the anoxic and euhaline medium of B30 sample, results indicate that these bacteria use their membrane transporters, both ions and peptides, to activate their cell signaling and defense system. This is in line with what Thureborn and colleagues (2013) found in the eutrophic and stratified waters of the Baltic sea. In those waters, same authors described that anoxic communities show comparatively higher abundances of genes related to the attachment to and utilization of organic material, including chemotaxis and motility (pilus formation), regulation and cell signaling and defense mechanism genes (quorum sensing and biofilm formation), degradation of polysaccharides and amino sugars and a comparatively lower abundance of fatty acid and secondary metabolism genes [56], in accordance to what was detected in the present study (Supplementary 5).

When analyzing the community and functional dissimilarities found between all the samples collected in this study, samples were classified similarly in all the methods/strategies used: CCA biplot based in bray distances (Fig. 4) and heatmap based on euclidean distances (Supplementary 1 and Supplementary 2). The highest bacterial community differences were shown when comparing riverine-freshwater samples (NER, GAL1) against saline water samples (B35, GAL2, B30) (Fig. 3). This result was similar to the findings in the amplicon sequencing study conducted in

this same estuary [12]. This result was explained by the contrasting environmental variables occurring between tributarine freshwaters and saline-oceanic ecosystems, being the latters less variable.

Interestingly, freshwater communities of each of the tributaries were dissimilar according to both, the taxonomic and functional analyses (Fig. 3 and Fig.4). This particularity might be due to the different intrinsic features of each of the rivers (orography, different soils in each basin or different sources of aquatic pollution).Nervion-Ibaizabal and Galindo showed its own characteristic bacterial community. On the one hand, in the sample of Nervion-Ibaizabal river (NER) the most abundant genus were related with freshwater bacteria: *Limnohabitans* [57], *Fluviicola* [58], *Flavobacterium* [51], *Rhodobacter* [59], etc. On the other hand, in the case of Galindo river, (GAL1 sample), some of the detected genus (e.g *Gordonia*, *Nitrosomonas*, *Rhodoferax*, *Chloroflexus*, *Bdellovibrio*, *Nitrospira*) were related with WWTP processes, as already described by other authors in other WWTP installations [16,17,60–62]. However, most of the taxa that were located in GAL1 were not detected in the rest of downstream estuarine samples (GAL2 and B35). This could be due to the low discharge of Galindo, which might not be enough to counter the tidal flux and freshwater mass would shift upwards when the tide rises, as occur in other estuaries [63], making the waters stagnant in the inner Galindo's basin.

Conclusions

In conclusion, while 16S rDNA amplicon technique allows predicting which microorganisms most likely are interacting with the environment, metagenomic and metatranscriptomic approaches uncover the active fraction of bacteria and the mechanisms they use to actually respond with the environment, allowing the understanding of the bacterial metabolic changes in the ecosystem. Thus, in ecosystems with pronounced environmental gradients, such as estuaries, it is possible to study how bacteria modify their metabolism to adapt to environmental changes. This was for instance evident for Limnohabitans bacterial genus in the Estuary of Bilbao. This bacteria activated the cellular movement and membrane transport proteins in eutrophic-saline waters, while it had these functions silenced in freshwater samples. Similarly, in the eutrophic water mass, the bacterial community modified its metabolism activating specific energy metabolism pathways (such as ethylmalonyl pathway, reductive acetyl-CoA pathway or isoprenoid biosynthesis) as to replenish intermediary metabolites (malate, succinate, isoprene or monoterpenes) and be able to continue with their metabolism allowing these bacteria to survive to hypoxia conditions.

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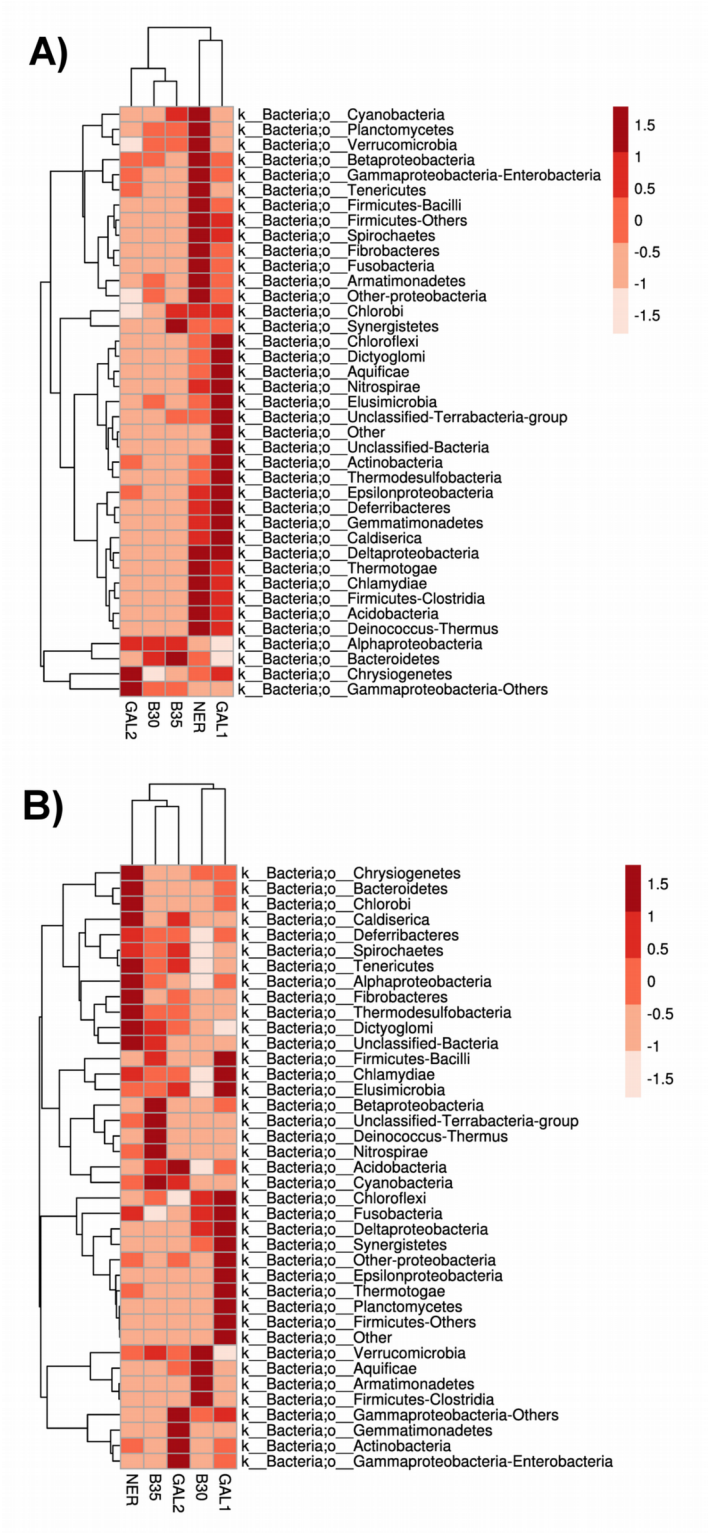
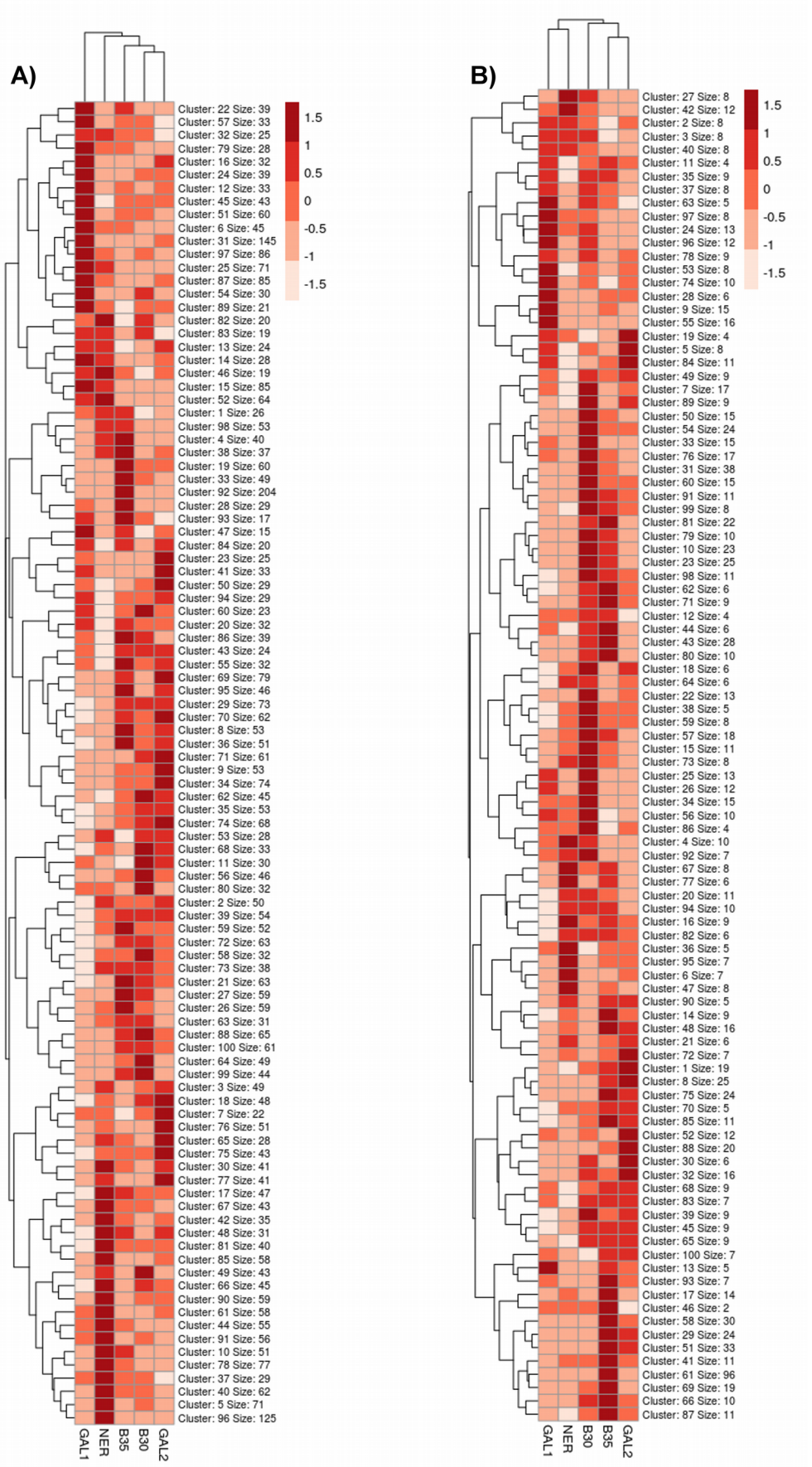
Supplementary material

Supplementary 1: Taxonomic-heatmap with the bacterial order distribution. A heatmap constructed with the taxonomic information (presence and abundance) extracted from metagenomic (A) and metatranscriptomic (B) sequence information obtained using GhostKOALA against KEGG database. The taxonomic category shown is at the order level.


A)
k__Bacteria;o__Cyanobacteria
k__Bacteria;o__Planctomycetes
k__Bacteria;o__Verrucomicrobia
k__Bacteria;o__Betaproteobacteria
k__Bacteria;o__Gammaproteobacteria-Enterobacteria
k__Bacteria;o__Tenericutes
k__Bacteria;o__Firmicutes-Bacilli
k__Bacteria;o__Firmicutes-Others
k__Bacteria;o__Spirochaetes
k__Bacteria;o__Fibrobacteres
k__Bacteria;o__Fusobacteria
k__Bacteria;o__Armatimonadetes
k__Bacteria;o__Other-proteobacteria
k__Bacteria;o__Chlorobi
k__Bacteria;o__Synergistetes
k__Bacteria;o__Chloroflexi
k__Bacteria;o__Dictyoglomi
k__Bacteria;o__Aquificae
k__Bacteria;o__Nitrospirae
k__Bacteria;o__Elusimicrobia
k__Bacteria;o__Unclassified-Terrabacteria-group
k__Bacteria;o__Other
k__Bacteria;o__Unclassified-Bacteria
k__Bacteria;o__Actinobacteria
k__Bacteria;o__Thermodesulfobacteria
k__Bacteria;o__Epsilonproteobacteria
k__Bacteria;o__Deferribacteres
k__Bacteria;o__Gemmatimonadetes
k__Bacteria;o__Caldiserica
k__Bacteria;o__Deltaproteobacteria
k__Bacteria;o__Thermotogae
k__Bacteria;o__Chlamydiae
k__Bacteria;o__Firmicutes-Clostridia
k__Bacteria;o__Acidobacteria
k__Bacteria;o__Deinococcus-Thermus
k__Bacteria;o__Alphaproteobacteria
k__Bacteria;o__Bacteroidetes
k__Bacteria;o__Chrysiogenetes
k__Bacteria;o__Gammaproteobacteria-Others
GAL2
B30
B35
NER
GAL1
1.5
1
0.5
0
-0.5
-1
-1.5
B)
k__Bacteria;o__Chrysiogenetes
k__Bacteria;o__Bacteroidetes
k__Bacteria;o__Chlorobi
k__Bacteria;o__Caldiserica
k__Bacteria;o__Deferribacteres
k__Bacteria;o__Spirochaetes
k__Bacteria;o__Tenericutes
k__Bacteria;o__Alphaproteobacteria
k__Bacteria;o__Fibrobacteres
k__Bacteria;o__Thermodesulfobacteria
k__Bacteria;o__Dictyoglomi
k__Bacteria;o__Unclassified-Bacteria
k__Bacteria;o__Firmicutes-Bacilli
k__Bacteria;o__Chlamydiae
k__Bacteria;o__Elusimicrobia
k__Bacteria;o__Betaproteobacteria
k__Bacteria;o__Unclassified-Terrabacteria-group
k__Bacteria;o__Deinococcus-Thermus
k__Bacteria;o__Nitrospirae
k__Bacteria;o__Acidobacteria
k__Bacteria;o__Cyanobacteria
k__Bacteria;o__Chloroflexi
k__Bacteria;o__Fusobacteria
k__Bacteria;o__Deltaproteobacteria
k__Bacteria;o__Synergistetes
k__Bacteria;o__Other-proteobacteria
k__Bacteria;o__Epsilonproteobacteria
k__Bacteria;o__Thermotogae
k__Bacteria;o__Planctomycetes
k__Bacteria;o__Firmicutes-Others
k__Bacteria;o__Other
k__Bacteria;o__Verrucomicrobia
k__Bacteria;o__Aquificae
k__Bacteria;o__Armatimonadetes
k__Bacteria;o__Firmicutes-Clostridia
k__Bacteria;o__Gammaproteobacteria-Others
k__Bacteria;o__Gemmatimonadetes
k__Bacteria;o__Actinobacteria
k__Bacteria;o__Gammaproteobacteria-Enterobacteria
NER
B35
GAL2
B30
GAL1
1.5
1
0.5
0
-0.5
-1
-1.5

Supplementary 2: Functional-heatmap based on KEGG Ortholog (KO) numbers. A heatmap constructed with the functional information (presence and abundance) extracted from metagenomic (A) and metatranscriptomic (B) sequence information obtained using GhostKOALA against KEGG database. KO functions were grouped at 100 clusters to construct each heatmap.


A)
Cluster: 22 Size: 39
Cluster: 57 Size: 33
Cluster: 32 Size: 25
Cluster: 79 Size: 28
Cluster: 16 Size: 32
Cluster: 24 Size: 39
Cluster: 12 Size: 33
Cluster: 45 Size: 43
Cluster: 51 Size: 60
Cluster: 6 Size: 45
Cluster: 31 Size: 145
Cluster: 97 Size: 86
Cluster: 25 Size: 71
Cluster: 87 Size: 85
Cluster: 54 Size: 30
Cluster: 89 Size: 21
Cluster: 82 Size: 20
Cluster: 83 Size: 19
Cluster: 13 Size: 24
Cluster: 14 Size: 28
Cluster: 46 Size: 19
Cluster: 15 Size: 85
Cluster: 52 Size: 64
Cluster: 1 Size: 26
Cluster: 98 Size: 53
Cluster: 4 Size: 40
Cluster: 38 Size: 37
Cluster: 19 Size: 60
Cluster: 33 Size: 49
Cluster: 92 Size: 204
Cluster: 28 Size: 29
Cluster: 93 Size: 17
Cluster: 47 Size: 15
Cluster: 84 Size: 20
Cluster: 23 Size: 25
Cluster: 41 Size: 33
Cluster: 50 Size: 29
Cluster: 94 Size: 29
Cluster: 60 Size: 23
Cluster: 20 Size: 32
Cluster: 86 Size: 39
Cluster: 43 Size: 24
Cluster: 55 Size: 32
Cluster: 69 Size: 79
Cluster: 95 Size: 46
Cluster: 29 Size: 73
Cluster: 70 Size: 62
Cluster: 8 Size: 53
Cluster: 36 Size: 51
Cluster: 71 Size: 61
Cluster: 9 Size: 53
Cluster: 34 Size: 74
Cluster: 62 Size: 45
Cluster: 35 Size: 53
Cluster: 74 Size: 68
Cluster: 53 Size: 28
Cluster: 68 Size: 33
Cluster: 11 Size: 30
Cluster: 56 Size: 46
Cluster: 80 Size: 32
Cluster: 2 Size: 50
Cluster: 39 Size: 54
Cluster: 59 Size: 52
Cluster: 72 Size: 63
Cluster: 58 Size: 32
Cluster: 73 Size: 38
Cluster: 21 Size: 63
Cluster: 27 Size: 59
Cluster: 26 Size: 59
Cluster: 63 Size: 31
Cluster: 88 Size: 65
Cluster: 100 Size: 61
Cluster: 64 Size: 49
Cluster: 99 Size: 44
Cluster: 3 Size: 49
Cluster: 18 Size: 48
Cluster: 7 Size: 22
Cluster: 76 Size: 51
Cluster: 65 Size: 28
Cluster: 75 Size: 43
Cluster: 30 Size: 41
Cluster: 77 Size: 41
Cluster: 17 Size: 47
Cluster: 67 Size: 43
Cluster: 42 Size: 35
Cluster: 48 Size: 31
Cluster: 81 Size: 40
Cluster: 85 Size: 58
Cluster: 49 Size: 43
Cluster: 66 Size: 45
Cluster: 90 Size: 59
Cluster: 61 Size: 58
Cluster: 44 Size: 55
Cluster: 91 Size: 56
Cluster: 10 Size: 51
Cluster: 78 Size: 77
Cluster: 37 Size: 29
Cluster: 40 Size: 62
Cluster: 5 Size: 71
Cluster: 96 Size: 125
1.5
1
0.5
0
-0.5
-1
-1.5
GAL1
NER
B35
B30
GAL2
B)
Cluster: 27 Size: 8
Cluster: 42 Size: 12
Cluster: 2 Size: 8
Cluster: 3 Size: 8
Cluster: 40 Size: 8
Cluster: 11 Size: 4
Cluster: 35 Size: 9
Cluster: 37 Size: 8
Cluster: 63 Size: 5
Cluster: 97 Size: 8
Cluster: 24 Size: 13
Cluster: 96 Size: 12
Cluster: 78 Size: 9
Cluster: 53 Size: 8
Cluster: 74 Size: 10
Cluster: 28 Size: 6
Cluster: 9 Size: 15
Cluster: 55 Size: 16
Cluster: 19 Size: 4
Cluster: 5 Size: 8
Cluster: 84 Size: 11
Cluster: 49 Size: 9
Cluster: 7 Size: 17
Cluster: 89 Size: 9
Cluster: 50 Size: 15
Cluster: 54 Size: 24
Cluster: 33 Size: 15
Cluster: 76 Size: 17
Cluster: 31 Size: 38
Cluster: 60 Size: 15
Cluster: 91 Size: 11
Cluster: 99 Size: 8
Cluster: 81 Size: 22
Cluster: 79 Size: 10
Cluster: 10 Size: 23
Cluster: 23 Size: 25
Cluster: 98 Size: 11
Cluster: 62 Size: 6
Cluster: 71 Size: 9
Cluster: 12 Size: 4
Cluster: 44 Size: 6
Cluster: 43 Size: 28
Cluster: 80 Size: 10
Cluster: 18 Size: 6
Cluster: 64 Size: 6
Cluster: 22 Size: 13
Cluster: 38 Size: 5
Cluster: 59 Size: 8
Cluster: 57 Size: 18
Cluster: 15 Size: 11
Cluster: 73 Size: 8
Cluster: 25 Size: 13
Cluster: 26 Size: 12
Cluster: 34 Size: 15
Cluster: 56 Size: 10
Cluster: 86 Size: 4
Cluster: 4 Size: 10
Cluster: 92 Size: 7
Cluster: 67 Size: 8
Cluster: 77 Size: 6
Cluster: 20 Size: 11
Cluster: 94 Size: 10
Cluster: 16 Size: 9
Cluster: 82 Size: 6
Cluster: 36 Size: 5
Cluster: 95 Size: 7
Cluster: 6 Size: 7
Cluster: 47 Size: 8
Cluster: 90 Size: 5
Cluster: 14 Size: 9
Cluster: 48 Size: 16
Cluster: 21 Size: 6
Cluster: 72 Size: 7
Cluster: 1 Size: 19
Cluster: 8 Size: 25
Cluster: 75 Size: 24
Cluster: 70 Size: 5
Cluster: 85 Size: 11
Cluster: 52 Size: 12
Cluster: 88 Size: 20
Cluster: 30 Size: 6
Cluster: 32 Size: 16
Cluster: 68 Size: 9
Cluster: 83 Size: 7
Cluster: 39 Size: 9
Cluster: 45 Size: 9
Cluster: 65 Size: 9
Cluster: 100 Size: 7
Cluster: 13 Size: 5
Cluster: 93 Size: 7
Cluster: 17 Size: 14
Cluster: 46 Size: 2
Cluster: 58 Size: 30
Cluster: 29 Size: 24
Cluster: 51 Size: 33
Cluster: 41 Size: 11
Cluster: 61 Size: 96
Cluster: 69 Size: 19
Cluster: 66 Size: 10
Cluster: 87 Size: 11
1.5
1
0.5
0
-0.5
-1
-1.5
GAL1
NER
B30
B35
GAL2

Supplementary 3: Characteristic KEGG modules of each sample. Shared and unique KEGG modules between and within samples. KEGG modules with the highest abundances (1st quartile) in the metatranscriptomic dataset were used.

Names	Total	Functions
B30 B35 GAL1 GAL2 NER	50	M00052 Pyrimidine ribonucleotide biosynthesis, UMP => UDP/UTP,CDP/CTP
		M00049 Adenine ribonucleotide biosynthesis, IMP => ADP,ATP
		M00532 Photorespiration
		M00157 F-type ATPase, prokaryotes and chloroplasts
		M00007 Pentose phosphate pathway, non-oxidative phase, fructose 6P => ribose 5P
		M00336 Twin-arginine translocation (Tat) system
		M00394 RNA degradosome
		M00165 Reductive pentose phosphate cycle (Calvin cycle)
		M00035 Methionine degradation
		M00173 Reductive citrate cycle (Arnon-Buchanan cycle)
		M00003 Gluconeogenesis, oxaloacetate => fructose-6P
		M00166 Reductive pentose phosphate cycle, ribulose-5P => glyceraldehyde-3P
		M00178 Ribosome, bacteria
		M00011 Citrate cycle, second carbon oxidation, 2-oxoglutarate => oxaloacetate
		M00083 Fatty acid biosynthesis, elongation
		M00151 Cytochrome bc1 complex respiratory unit
		M00620 Incomplete reductive citrate cycle, acetyl-CoA => oxoglutarate
		M00012 Glyoxylate cycle
		M00155 Cytochrome c oxidase, prokaryotes
		M00374 Dicarboxylate-hydroxybutyrate cycle
		M00053 Pyrimidine deoxyribonuleotide biosynthesis, CDP/CTP => dCDP/dCTP,dTDP/dTTP
		M00050 Guanine ribonucleotide biosynthesis IMP => GDP,GTP
		M00167 Reductive pentose phosphate cycle, glyceraldehyde-3P => ribulose-5P
		M00179 Ribosome, archaea
		M00742 Aminoglycoside resistance, protease FtsH
		M00570 Isoleucine biosynthesis, threonine => 2-oxobutanoate => isoleucine
		M00144 NADH:quinone oxidoreductase, prokaryotes
		M00149 Succinate dehydrogenase, prokaryotes
		M00002 Glycolysis, core module involving three-carbon compounds
		M00161 Photosystem II
		M00183 RNA polymerase, bacteria
		M00552 D-galactonate degradation, De Ley-Doudoroff pathway, D-galactonate => glycerate-3P
		M00335 Sec (secretion) system
		M00162 Cytochrome b6f complex
		M00001 Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate
		M00740 Methylaspartate cycle
		M00572 Pimeloyl-ACP biosynthesis, BioC-BioH pathway, malonyl-ACP => pimeloyl-ACP
		M00307 Pyruvate oxidation, pyruvate => acetyl-CoA
		M00010 Citrate cycle, first carbon oxidation, oxaloacetate => 2-oxoglutarate
		M00005 PRPP biosynthesis, ribose 5P => PRPP
		M00009 Citrate cycle (TCA cycle, Krebs cycle)
		M00152 Cytochrome bc1 complex
		M00004 Pentose phosphate pathway (Pentose phosphate cycle)
		M00019 Valine/isoleucine biosynthesis, pyruvate => valine / 2-oxobutanoate => isoleucine
		M00346 Formaldehyde assimilation, serine pathway
		M00163 Photosystem I
		M00308 Semi-phosphorylative Entner-Doudoroff pathway, gluconate => glycerate-3P
		M00344 Formaldehyde assimilation, xylulose monophosphate pathway
		M00376 3-Hydroxypropionate bi-cycle
		M00345 Formaldehyde assimilation, ribulose monophosphate pathway
B30 B35 GAL1 NER	2	M00237 Branched-chain amino acid transport system
		M00221 Putative simple sugar transport system
B30 B35 GAL2 NER	11	M00022 Shikimate pathway, phosphoenolpyruvate + erythrose-4P => chorismate
		M00609 Cysteine biosynthesis, methionine => cysteine
		M00126 Tetrahydrofolate biosynthesis, GTP => THF
		M00034 Methionine salvage pathway
		M00125 Riboflavin biosynthesis, GTP => riboflavin/FMN/FAD
		M00140 C1-unit interconversion, prokaryotes
		M00222 Phosphate transport system
		M00051 Uridine monophosphate biosynthesis, glutamine (+ PRPP) => UMP
		M00121 Heme biosynthesis, glutamate => protoheme/siroheme
		M00565 Trehalose biosynthesis, D-glucose 1P => trehalose
		M00368 Ethylene biosynthesis, methionine => ethylene
B30 B35 GAL1 GAL2	1	M00026 Histidine biosynthesis, PRPP => histidine
B30 GAL1 GAL2 NER	3	M00048 Inosine monophosphate biosynthesis, PRPP + glutamine => IMP
		M00168 CAM (Crassulacean acid metabolism), dark
		M00036 Leucine degradation, leucine => acetoacetate + acetyl-CoA
B30 B35 NER	3	M00841 Tetrahydrofolate biosynthesis, mediated by PTPS, GTP => THF
		M00843 L-threo-Tetrahydrobiopterin biosynthesis, GTP => L-threo-BH4
		M00842 Tetrahydrobiopterin biosynthesis, GTP => BH4

B30 B35 GAL2	**7**	M00060 Lipopolysaccharide biosynthesis, KDO2-lipid A
		M00596 Dissimilatory sulfate reduction, sulfate => H2S
		M00176 Assimilatory sulfate reduction, sulfate => H2S
		M00236 Putative polar amino acid transport system
		M00207 Putative multiple sugar transport system
		M00232 General L-amino acid transport system
		M00208 Glycine betaine/proline transport system
B30 GAL2 NER	**4**	M00258 Putative ABC transport system
		M00082 Fatty acid biosynthesis, initiation
		M00032 Lysine degradation, lysine => saccharopine => acetoacetyl-CoA
		M00119 Pantothenate biosynthesis, valine/L-aspartate => pantothenate
B35 GAL2 NER	**3**	M00362 Nucleotide sugar biosynthesis, prokaryotes
		M00017 Methionine biosynthesis, apartate => homoserine => methionine
		M00124 Pyridoxal biosynthesis, erythrose-4P => pyridoxal-5P
GAL1 GAL2 NER	**1**	M00018 Threonine biosynthesis, aspartate => homoserine => threonine
B30 B35	**3**	M00523 RegB-RegA (redox response) two-component regulatory system
		M00201 alpha-Glucoside transport system
		M00120 Coenzyme A biosynthesis, pantothenate => CoA
B30 NER	**5**	M00095 C5 isoprenoid biosynthesis, mevalonate pathway
		M00096 C5 isoprenoid biosynthesis, non-mevalonate pathway
		M00089 Triacylglycerol biosynthesis
		M00365 C10-C20 isoprenoid biosynthesis, archaea
		M00364 C10-C20 isoprenoid biosynthesis, bacteria
		M00840 Tetrahydrofolate biosynthesis, mediated by ribA and trpF, GTP => THF
		M00334 Type VI secretion system
B30 GAL2	**5**	M00728 Cationic antimicrobial peptide (CAMP) resistance, protein folding and degrading factors DegP and DsbA
		M00729 Fluoroquinolone resistance, gyrase-protecting protein Qnr
		M00580 Pentose phosphate pathway, archaea, fructose 6P => ribose 5P
		M00190 Iron(III) transport system
		M00212 Ribose transport system
B35 NER	**1**	M00186 Tungstate transport system
B35 GAL1	**1**	M00209 Osmoprotectant transport system
B35 GAL2	**4**	M00014 Glucuronate pathway (uronate pathway)
		M00129 Ascorbate biosynthesis, animals, glucose-1P => ascorbate
		M00360 Aminoacyl-tRNA biosynthesis, prokaryotes
		M00254 ABC-2 type transport system
GAL2 NER	**11**	M00016 Lysine biosynthesis, succinyl-DAP pathway, aspartate => lysine
		M00525 Lysine biosynthesis, acetyl-DAP pathway, aspartate => lysine
		M00357 Methanogenesis, acetate => methane
		M00029 Urea cycle
		M00526 Lysine biosynthesis, DAP dehydrogenase pathway, aspartate => lysine
		M00020 Serine biosynthesis, glycerate-3P => serine
		M00527 Lysine biosynthesis, DAP aminotransferase pathway, aspartate => lysine
		M00116 Menaquinone biosynthesis, chorismate => menaquinone
		M00033 Ectoine biosynthesis, aspartate => ectoine
		M00529 Denitrification, nitrate => nitrogen
		M00506 CheA-CheYBV (chemotaxis) two-component regulatory system
GAL1 GAL2	**2**	M00154 Cytochrome c oxidase
		M00804 Complete nitrification, comammox, ammonia => nitrite => nitrate
B30	**12**	M00528 Nitrification, ammonia => nitrite
		M00021 Cysteine biosynthesis, serine => cysteine
		M00215 D-Xylose transport system
		M00174 Methane oxidation, methanotroph, methane => formaldehyde
		M00156 Cytochrome c oxidase, cbb3-type
		M00373 Ethylmalonyl pathway
		M00239 Peptides/nickel transport system
		M00377 Reductive acetyl-CoA pathway (Wood-Ljungdahl pathway)
		M00088 Ketone body biosynthesis, acetyl-CoA => acetoacetate/3-hydroxybutyrate/acetone
		M00375 Hydroxypropionate-hydroxybutylate cycle
		M00193 Putative spermidine/putrescine transport system
		M00097 beta-Carotene biosynthesis, GGAP => beta-carotene
B35	**5**	M00607 Glycerol transport system
		M00240 Iron complex transport system
		M00280 PTS system, glucitol/sorbitol-specific II component
		M00008 Entner-Doudoroff pathway, glucose-6P => glyceraldehyde-3P + pyruvate
		M00822 Multidrug resistance, efflux pump MexMN-OprM
NER	**19**	M00046 Pyrimidine degradation, uracil => beta-alanine, thymine => 3-aminoisobutanoate
		M00615 Nitrate assimilation
		M00642 Multidrug resistance, efflux pump MexJK-OprM
		M00024 Phenylalanine biosynthesis, chorismate => phenylalanine
		M00025 Tyrosine biosynthesis, chorismate => tyrosine
		M00045 Histidine degradation, histidine => N-formiminoglutamate => glutamate
		M00006 Pentose phosphate pathway, oxidative phase, glucose 6P => ribulose 5P
		M00189 Molybdate transport system
		M00260 DNA polymerase III complex, bacteria
		M00718 Multidrug resistance, efflux pump MexAB-OprM
		M00038 Tryptophan metabolism, tryptophan => kynurenine => 2-aminomuconate
		M00027 GABA (gamma-Aminobutyrate) shunt
		M00643 Multidrug resistance, efflux pump MexXY-OprM
		M00768 Multidrug resistance, efflux pump GesABC
		M00549 Nucleotide sugar biosynthesis, glucose => UDP-glucose
		M00647 Multidrug resistance, efflux pump AcrAB-TolC/SmeDEF
		M00434 PhoR-PhoB (phosphate starvation response) two-component regulatory system

		M00150 Fumarate reductase, prokaryotes
		M00331 Type II general secretion pathway
		M00122 Cobalamin biosynthesis, cobinamide => cobalamin
GAL1	**3**	M00328 Hemophore/metalloprotease transport system
		M00432 Leucine biosynthesis, 2-oxoisovalerate => 2-oxoisocaproate
		M00743 Aminoglycoside resistance, protease HtpX
GAL2	**11**	M00170 C4-dicarboxylic acid cycle, phosphoenolpyruvate carboxykinase type
		M00015 Proline biosynthesis, glutamate => proline
		M00117 Ubiquinone biosynthesis, prokaryotes, chorismate => ubiquinone
		M00086 beta-Oxidation, acyl-CoA synthesis
		M00023 Tryptophan biosynthesis, chorismate => tryptophan
		M00171 C4-dicarboxylic acid cycle, NAD - malic enzyme type
		M00811 Nicotine degradation, pyrrolidine pathway, nicotine => succinate semialdehyde
		M00172 C4-dicarboxylic acid cycle, NADP - malic enzyme type

Supplementary 4: The most active functions of each sample. List of the most abundant KEGG Orhtologs (KO) within sample type (NER, B30, B35,GAL1, GAL2). Only abundances higher 1% were taken into account.

NER

ko01000 Enzymes(7)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]
K02548 menA; 1,4-dihydroxy-2-naphthoate octaprenyltransferase [EC:2.5.1.74 2.5.1.-]
K11749 rseP; regulator of sigma E protease [EC:3.4.24.-]
K01464 DPYS; dihydropyrimidinase [EC:3.5.2.2]
K01599 hemE; uroporphyrinogen decarboxylase [EC:4.1.1.37]
K01602 rbcS; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]
K16329 psuG; pseudouridylate synthase [EC:4.2.1.70]

ko00194 Photosynthesis proteins(6)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]
K02691 psaC; photosystem I subunit VII
K02697 psaJ; photosystem I subunit IX
K02639 petF; ferredoxin
K02110 ATPF0C; F-type H+-transporting ATPase subunit c
K02108 ATPF0A; F-type H+-transporting ATPase subunit a

ko02000 Transporters(1)

K18139 oprM; outer membrane protein, multidrug efflux system

ko01006 Prenyltransferases(1)

K02548 menA; 1,4-dihydroxy-2-naphthoate octaprenyltransferase [EC:2.5.1.74 2.5.1.-]

ko01002 Peptidases(1)

K11749 rseP; regulator of sigma E protease [EC:3.4.24.-]

ko04147 Exosome(1)

K01464 DPYS; dihydropyrimidinase [EC:3.5.2.2]

ko01504 Antimicrobial resistance genes(1)

K18139 oprM; outer membrane protein, multidrug efflux system

ko03110 Chaperones and folding catalysts(1)

K02108 ATPF0A; F-type H+-transporting ATPase subunit a

B30

ko01000 Enzymes(7)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]
K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]
K01126 E3.1.4.46; glycerophosphoryl diester phosphodiesterase [EC:3.1.4.46]
K01601 rbcL; ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]
K01602 rbcS; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]
K01682 acnB; aconitate hydratase 2 / 2-methylisocitrate dehydratase [EC:4.2.1.3 4.2.1.99]
K01912 paaK; phenylacetate-CoA ligase [EC:6.2.1.30]

ko00194 Photosynthesis proteins(6)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]
K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]
K02639 petF; ferredoxin
K02110 ATPF0C; F-type H+-transporting ATPase subunit c

K02108 ATPF0A; F-type H+-transporting ATPase subunit a

K05377 cpeB; phycoerythrin beta chain

ko03110 Chaperones and folding catalysts(1)

K02108 ATPF0A; F-type H+-transporting ATPase subunit a

B35

ko00194 Photosynthesis proteins(11)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]

K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]

K02705 psbC; photosystem II CP43 chlorophyll apoprotein

K02709 psbH; photosystem II PsbH protein

K02712 psbK; photosystem II PsbK protein

K02692 psaD; photosystem I subunit II

K02637 petD; cytochrome b6-f complex subunit 4

K02639 petF; ferredoxin

K02109 ATPF0B; F-type H+-transporting ATPase subunit b

K02110 ATPF0C; F-type H+-transporting ATPase subunit c

K02108 ATPF0A; F-type H+-transporting ATPase subunit a

ko01000 Enzymes(7)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]

K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]

K11753 ribF; riboflavin kinase / FMN adenylyltransferase [EC:2.7.1.26 2.7.7.2]

K01000 mraY; phospho-N-acetylmuramoyl-pentapeptide-transferase [EC:2.7.8.13]

K03106 SRP54; signal recognition particle subunit SRP54 [EC:3.6.5.4]

K01601 rbcL; ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]

K01602 rbcS; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]

ko01011 Peptidoglycan biosynthesis and degradation proteins(1)

K01000 mraY; phospho-N-acetylmuramoyl-pentapeptide-transferase [EC:2.7.8.13]

ko03110 Chaperones and folding catalysts(1)

K02108 ATPF0A; F-type H+-transporting ATPase subunit a

ko03012 Translation factors(1)

K02837 prfC; peptide chain release factor 3

ko02044 Secretion system(1)

K03106 SRP54; signal recognition particle subunit SRP54 [EC:3.6.5.4]

GAL1

ko01000 Enzymes(8)

K04090 E1.2.7.8; indolepyruvate ferredoxin oxidoreductase [EC:1.2.7.8]

K02274 coxA; cytochrome c oxidase subunit I [EC:1.9.3.1]

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]

K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]

K00615 E2.2.1.1; transketolase [EC:2.2.1.1]

K01575 alsD; acetolactate decarboxylase [EC:4.1.1.5]

K01601 rbcL; ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]

K01602 rbcS; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]

ko00194 Photosynthesis proteins(3)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]

K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]

K02110 ATPF0C; F-type H+-transporting ATPase subunit c

ko03011 Ribosome(1)

K02935 RP-L7; large subunit ribosomal protein L7/L12

ko02000 Transporters(1)

K05568 mnhD; multicomponent Na+:H+ antiporter subunit D

GAL2

ko00194 Photosynthesis proteins(11)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]

K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]

K02705 psbC; photosystem II CP43 chlorophyll apoprotein

K02712 psbK; photosystem II PsbK protein

K02692 psaD; photosystem I subunit II

K02637 petD; cytochrome b6-f complex subunit 4

K02639 petF; ferredoxin

K02111 ATPF1A; F-type H+-transporting ATPase subunit alpha [EC:3.6.3.14]

K02109 ATPF0B; F-type H+-transporting ATPase subunit b

K02110 ATPF0C; F-type H+-transporting ATPase subunit c

K02108 ATPF0A; F-type H+-transporting ATPase subunit a

ko01000 Enzymes(6)

K02703 psbA; photosystem II P680 reaction center D1 protein [EC:1.10.3.9]

K02706 psbD; photosystem II P680 reaction center D2 protein [EC:1.10.3.9]

K00931 proB; glutamate 5-kinase [EC:2.7.2.11]

K02111 ATPF1A; F-type H+-transporting ATPase subunit alpha [EC:3.6.3.14]

K01601 rbcL; ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]

K01602 rbcS; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]

ko03110 Chaperones and folding catalysts(1)

K02108 ATPF0A; F-type H+-transporting ATPase subunit a

ko03021 Transcription machinery(1)

K03092 SIG54; RNA polymerase sigma-54 factor

Supplementary 5: The most active functions (KO) of B30 and their cluster number. List of the KO information associated to the cluster numbers in Figure 5.

Cluster	KO Nº	KO object
50	K00995	pgsA; CDP-diacylglycerol---glycerol-3-phosphate 3-phosphatidyltransferase [EC:2.7.8.5]
50	K03775	slyD; FKBP-type peptidyl-prolyl cis-trans isomerase SlyD [EC:5.2.1.8]
50	K06603	flaG; flagellar protein FlaG
50	K07390	grxD; monothiol glutaredoxin
50	K09748	rimP; ribosome maturation factor RimP
49	K02357	tsf; elongation factor Ts
49	K04043	dnaK; molecular chaperone DnaK
48	K00252	GCDH; glutaryl-CoA dehydrogenase [EC:1.3.8.6]
48	K00340	nuoK; NADH-quinone oxidoreductase subunit K [EC:1.6.5.3]
48	K00341	nuoL; NADH-quinone oxidoreductase subunit L [EC:1.6.5.3]
48	K00507	SCD; stearoyl-CoA desaturase (Delta-9 desaturase) [EC:1.14.19.1]
48	K00573	E2.1.1.77; protein-L-isoaspartate(D-aspartate) O-methyltransferase [EC:2.1.1.77]
48	K07101	uncharacterized protein
47	K06911	uncharacterized protein
46	K02058	ABC.SS.S; simple sugar transport system substrate-binding protein
46	K17225	soxC; sulfane dehydrogenase subunit SoxC
46	K00340.K05560	nuoK; NADH-quinone oxidoreductase subunit K [EC:1.6.5.3]
46	K00605	gcvT; aminomethyltransferase [EC:2.1.2.10]
46	K01951	guaA; GMP synthase (glutamine-hydrolysing) [EC:6.3.5.2]
46	K02049	ABC.SN.A; NitT/TauT family transport system ATP-binding protein
46	K02111	ATPF1A; F-type H+-transporting ATPase subunit alpha [EC:3.6.3.14]
46	K02372	fabZ; 3-hydroxyacyl-[acyl-carrier-protein] dehydratase [EC:4.2.1.59]
46	K02437	gcvH; glycine cleavage system H protein
46	K02520	infC; translation initiation factor IF-3
46	K02864	RP-L10; large subunit ribosomal protein L10
46	K02887	RP-L20; large subunit ribosomal protein L20
46	K03320	amt; ammonium transporter, Amt family
46	K03407	cheA; two-component system, chemotaxis family, sensor kinase CheA [EC:2.7.13.3]
46	K03553	recA; recombination protein RecA
46	K03695	clpB; ATP-dependent Clp protease ATP-binding subunit ClpB
46	K03977	engA; GTPase
46	K04752	glnK; nitrogen regulatory protein P-II 2
46	K09158	uncharacterized protein
45	K02400	flhA; flagellar biosynthesis protein FlhA
45	K02892	RP-L23; large subunit ribosomal protein L23
45	K02906	RP-L3; large subunit ribosomal protein L3
45	K02948	RP-S11; small subunit ribosomal protein S11
45	K02992	RP-S7; small subunit ribosomal protein S7
44	K02422	fliS; flagellar protein FliS
44	K13643	iscR; Rrf2 family transcriptional regulator, iron-sulfur cluster assembly transcription factor
44	K01637	E4.1.3.1; isocitrate lyase [EC:4.1.3.1]
44	K02902	RP-L28; large subunit ribosomal protein L28
44	K02950	RP-S12; small subunit ribosomal protein S12
44	K03070	secA; preprotein translocase subunit SecA
44	K05838	ybbN; putative thioredoxin
43	K02838	frr; ribosome recycling factor
43	K02907	RP-L30; large subunit ribosomal protein L30
43	K03046	rpoC; DNA-directed RNA polymerase subunit beta' [EC:2.7.7.6]
43	K03217	yidC; YidC/Oxa1 family membrane protein insertase
43	K03686	dnaJ; molecular chaperone DnaJ
43	K04079	HSP90A; molecular chaperone HtpG
43	K06207	typA; GTP-binding protein
42	K00240	sdhB; succinate dehydrogenase / fumarate reductase, iron-sulfur subunit [EC:1.3.5.1 1.3.5.4]
42	K00615	E2.2.1.1; transketolase [EC:2.2.1.1]
42	K01495	GCH1; GTP cyclohydrolase IA [EC:3.5.4.16]
42	K03596	lepA; GTP-binding protein LepA
42	K03768	PPIB; peptidyl-prolyl cis-trans isomerase B (cyclophilin B) [EC:5.2.1.8]
41	K00799	GST; glutathione S-transferase [EC:2.5.1.18]
41	K01462	PDF; peptide deformylase [EC:3.5.1.88]
41	K01803	TPI; triosephosphate isomerase (TIM) [EC:5.3.1.1]
41	K02274	coxA; cytochrome c oxidase subunit I [EC:1.9.3.1]
41	K03611	dsbB; disulfide bond formation protein DsbB
41	K03685	rnc; ribonuclease III [EC:3.1.26.3]
41	K07399	resB; cytochrome c biogenesis protein
40	K00648	fabH; 3-oxoacyl-[acyl-carrier-protein] synthase III [EC:2.3.1.180]
40	K01338	lon; ATP-dependent Lon protease [EC:3.4.21.53]

40 K02358 tuf; elongation factor Tu
40 K02956 RP-S15; small subunit ribosomal protein S15
40 K02961 RP-S17; small subunit ribosomal protein S17
40 K02963 RP-S18; small subunit ribosomal protein S18
40 K02986 RP-S4; small subunit ribosomal protein S4
40 K02994 RP-S8; small subunit ribosomal protein S8
40 K02996 RP-S9; small subunit ribosomal protein S9
39 K00927.K01803 PGK; phosphoglycerate kinase [EC:2.7.2.3]
39 K01601 rbcL; ribulose-bisphosphate carboxylase large chain [EC:4.1.1.39]
39 K02258 COX11; cytochrome c oxidase assembly protein subunit 11
39 K00019 E1.1.1.30; 3-hydroxybutyrate dehydrogenase [EC:1.1.1.30]
39 K00023 phbB; acetoacetyl-CoA reductase [EC:1.1.1.36]
39 K00029 E1.1.1.40; malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+) [EC:1.1.1.40]
39 K00058 serA; D-3-phosphoglycerate dehydrogenase / 2-oxoglutarate reductase [EC:1.1.1.95 1.1.1.399]
39 K00067.K01790 rfbD; dTDP-4-dehydrorhamnose reductase [EC:1.1.1.133]
39 K00074 3-hydroxybutyryl-CoA dehydrogenase [EC:1.1.1.157]
39 K00123 fdoG; formate dehydrogenase major subunit [EC:1.17.1.9]
39 K00127 fdoI; formate dehydrogenase subunit gamma
39 K00140 mmsA; malonate-semialdehyde dehydrogenase (acetylating) / methylmalonate-semialdehyde dehydrogenase
39 K00228 CPOX; coproporphyrinogen III oxidase [EC:1.3.3.3]
39 K00266 gltD; glutamate synthase (NADPH/NADH) small chain [EC:1.4.1.13 1.4.1.14]
39 K00285 dadA; D-amino-acid dehydrogenase [EC:1.4.5.1]
39 K00311 ETFDH; electron-transferring-flavoprotein dehydrogenase [EC:1.5.5.1]
39 K00324 pntA; NAD(P) transhydrogenase subunit alpha [EC:1.6.1.2]
39 K00457 HPD; 4-hydroxyphenylpyruvate dioxygenase [EC:1.13.11.27]
39 K00528 fpr; ferredoxin/flavodoxin---NADP+ reductase [EC:1.18.1.2 1.19.1.1]
39 K00560 thyA; thymidylate synthase [EC:2.1.1.45]
39 K00575 cheR; chemotaxis protein methyltransferase CheR [EC:2.1.1.80]
39 K00640 cysE; serine O-acetyltransferase [EC:2.3.1.30]
39 K00641 metX; homoserine O-acetyltransferase [EC:2.3.1.31]
39 K00645 fabD; [acyl-carrier-protein] S-malonyltransferase [EC:2.3.1.39]
39 K00666 fatty-acyl-CoA synthase [EC:6.2.1.-]
39 K00681 ggt; gamma-glutamyltranspeptidase / glutathione hydrolase [EC:2.3.2.2 3.4.19.13]
39 K00764 purF; amidophosphoribosyltransferase [EC:2.4.2.14]
39 K00789 metK; S-adenosylmethionine synthetase [EC:2.5.1.6]
39 K00798 MMAB; cob(I)alamin adenosyltransferase [EC:2.5.1.17]
39 K00806 uppS; undecaprenyl diphosphate synthase [EC:2.5.1.31]
39 K00822 E2.6.1.18; beta-alanine--pyruvate transaminase [EC:2.6.1.18]
39 K00939 adk; adenylate kinase [EC:2.7.4.3]
39 K00942 E2.7.4.8; guanylate kinase [EC:2.7.4.8]
39 K00974 cca; tRNA nucleotidyltransferase (CCA-adding enzyme) [EC:2.7.7.72 3.1.3.- 3.1.4.-]
39 K01007 pps; pyruvate, water dikinase [EC:2.7.9.2]
39 K01061 E3.1.1.45; carboxymethylenebutenolidase [EC:3.1.1.45]
39 K01069 gloB; hydroxyacylglutathione hydrolase [EC:3.1.2.6]
39 K01255 CARP; leucyl aminopeptidase [EC:3.4.11.1]
39 K01356 lexA; repressor LexA [EC:3.4.21.88]
39 K01451 hipO; hippurate hydrolase [EC:3.5.1.32]
39 K01496 hisI; phosphoribosyl-AMP cyclohydrolase [EC:3.5.4.19]
39 K01520 dut; dUTP pyrophosphatase [EC:3.6.1.23]
39 K01524 ppx-gppA; exopolyphosphatase / guanosine-5'-triphosphate,3'-diphosphate pyrophosphatase [EC:3.6.1.11 3.6.1.40]
39 K01599 hemE; uroporphyrinogen decarboxylase [EC:4.1.1.37]
39 K01602 rbcS; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39]
39 K01625 eda; 2-dehydro-3-deoxyphosphogluconate aldolase / (4S)-4-hydroxy-2-oxoglutarate aldolase [EC:4.1.2.14 4.1.3.42]
39 K01653 E2.2.1.6S; acetolactate synthase I/III small subunit [EC:2.2.1.6]
39 K01662 dxs; 1-deoxy-D-xylulose-5-phosphate synthase [EC:2.2.1.7]
39 K01669 phrB; deoxyribodipyrimidine photo-lyase [EC:4.1.99.3]
39 K01698 hemB; porphobilinogen synthase [EC:4.2.1.24]
39 K01703 leuC; 3-isopropylmalate/(R)-2-methylmalate dehydratase large subunit [EC:4.2.1.33 4.2.1.35]
39 K01714 dapA; 4-hydroxy-tetrahydrodipicolinate synthase [EC:4.3.3.7]
39 K01736 aroC; chorismate synthase [EC:4.2.3.5]
39 K01754 E4.3.1.19; threonine dehydratase [EC:4.3.1.19]
39 K01759 GLO1; lactoylglutathione lyase [EC:4.4.1.5]
39 K01760 metC; cystathionine beta-lyase [EC:4.4.1.8]
39 K01783 rpe; ribulose-phosphate 3-epimerase [EC:5.1.3.1]
39 K01784 galE; UDP-glucose 4-epimerase [EC:5.1.3.2]
39 K01790 rfbC; dTDP-4-dehydrorhamnose 3,5-epimerase [EC:5.1.3.13]
39 K01814 hisA; phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase [EC:5.3.1.16]
39 K01845 hemL; glutamate-1-semialdehyde 2,1-aminomutase [EC:5.4.3.8]
39 K01875 SARS; seryl-tRNA synthetase [EC:6.1.1.11]
39 K01878 glyQ; glycyl-tRNA synthetase alpha chain [EC:6.1.1.14]
39 K01883 CARS; cysteinyl-tRNA synthetase [EC:6.1.1.16]
39 K01890 FARSB; phenylalanyl-tRNA synthetase beta chain [EC:6.1.1.20]
39 K01895 ACSS; acetyl-CoA synthetase [EC:6.2.1.1]

39	K01897	ACSL; long-chain acyl-CoA synthetase [EC:6.2.1.3]
39	K01912	paaK; phenylacetate-CoA ligase [EC:6.2.1.30]
39	K01924	murC; UDP-N-acetylmuramate--alanine ligase [EC:6.3.2.8]
39	K01962	accA; acetyl-CoA carboxylase carboxyl transferase subunit alpha [EC:6.4.1.2]
39	K01963	accD; acetyl-CoA carboxylase carboxyl transferase subunit beta [EC:6.4.1.2]
39	K01995	livG; branched-chain amino acid transport system ATP-binding protein
39	K01996	livF; branched-chain amino acid transport system ATP-binding protein
39	K01997	livH; branched-chain amino acid transport system permease protein
39	K01998	livM; branched-chain amino acid transport system permease protein
39	K02003	ABC.CD.A; putative ABC transport system ATP-binding protein
39	K02012	afuA; iron(III) transport system substrate-binding protein
39	K02025	ABC.MS.P; multiple sugar transport system permease protein
39	K02026	ABC.MS.P1; multiple sugar transport system permease protein
39	K02027	ABC.MS.S; multiple sugar transport system substrate-binding protein
39	K02033	ABC.PE.P; peptide/nickel transport system permease protein
39	K02034	ABC.PE.P1; peptide/nickel transport system permease protein
39	K02035	ABC.PE.S; peptide/nickel transport system substrate-binding protein
39	K02038	pstA; phosphate transport system permease protein
39	K02041	phnC; phosphonate transport system ATP-binding protein [EC:3.6.3.28]
39	K02044	phnD; phosphonate transport system substrate-binding protein
39	K02050	ABC.SN.P; NitT/TauT family transport system permease protein
39	K02065	mlaF; phospholipid/cholesterol/gamma-HCH transport system ATP-binding protein
39	K02067	mlaD; phospholipid/cholesterol/gamma-HCH transport system substrate-binding protein
39	K02198	ccmF; cytochrome c-type biogenesis protein CcmF
39	K02199	ccmG; cytochrome c biogenesis protein CcmG, thiol:disulfide interchange protein DsbE
39	K02200	ccmH; cytochrome c-type biogenesis protein CcmH
39	K02227	cbiB; adenosylcobinamide-phosphate synthase [EC:6.3.1.10]
39	K02337	dnaE; DNA polymerase III subunit alpha [EC:2.7.7.7]
39	K02343	dnaX; DNA polymerase III subunit gamma/tau [EC:2.7.7.7]
39	K02371	fabK; enoyl-[acyl-carrier protein] reductase II [EC:1.3.1.9]
39	K02390	flgE; flagellar hook protein FlgE
39	K02392	flgG; flagellar basal-body rod protein FlgG
39	K02393	flgH; flagellar L-ring protein precursor FlgH
39	K02395	flgJ; flagellar protein FlgJ
39	K02396	flgK; flagellar hook-associated protein 1 FlgK
39	K02407	fliD; flagellar hook-associated protein 2
39	K02452	gspC; general secretion pathway protein C
39	K02453	gspD; general secretion pathway protein D
39	K02456	gspG; general secretion pathway protein G
39	K02470	gyrB; DNA gyrase subunit B [EC:5.99.1.3]
39	K02502	hisZ; ATP phosphoribosyltransferase regulatory subunit
39	K02523	ispB; octaprenyl-diphosphate synthase [EC:2.5.1.90]
39	K02613	paaE; ring-1,2-phenylacetyl-CoA epoxidase subunit PaaE
39	K02650	pilA; type IV pilus assembly protein PilA
39	K02871	RP-L13; large subunit ribosomal protein L13
39	K02919	RP-L36; large subunit ribosomal protein L36
39	K02952	RP-S13; small subunit ribosomal protein S13
39	K03074	secF; preprotein translocase subunit SecF
39	K03088	SIG3.2; RNA polymerase sigma-70 factor, ECF subfamily
39	K03111	ssb; single-strand DNA-binding protein
39	K03118	tatC; sec-independent protein translocase protein TatC
39	K03218	rlmB; 23S rRNA (guanosine2251-2'-O)-methyltransferase [EC:2.1.1.185]
39	K03269	lpxH; UDP-2,3-diacylglucosamine hydrolase [EC:3.6.1.54]
39	K03412	cheB; two-component system, chemotaxis family, protein-glutamate methylesterase/glutaminase
39	K03466	ftsK; DNA segregation ATPase FtsK/SpoIIIE, S-DNA-T family
39	K03496	parA; chromosome partitioning protein
39	K03526	gcpE; (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase [EC:1.17.7.1 1.17.7.3]
39	K03527	ispH; 4-hydroxy-3-methylbut-2-en-1-yl diphosphate reductase [EC:1.17.7.4]
39	K03529	smc; chromosome segregation protein
39	K03585	acrA; membrane fusion protein, multidrug efflux system
39	K03593	mrp; ATP-binding protein involved in chromosome partitioning
39	K03600	sspB; stringent starvation protein B
39	K03602	xseB; exodeoxyribonuclease VII small subunit [EC:3.1.11.6]
39	K03608	minE; cell division topological specificity factor
39	K03704	cspA; cold shock protein (beta-ribbon, CspA family)
39	K03710	GntR family transcriptional regulator
39	K03711	fur; Fur family transcriptional regulator, ferric uptake regulator
39	K03738	aor; aldehyde:ferredoxin oxidoreductase [EC:1.2.7.5]
39	K03774	slpA; FKBP-type peptidyl-prolyl cis-trans isomerase SlpA [EC:5.2.1.8]
39	K03797	E3.4.21.102; carboxyl-terminal processing protease [EC:3.4.21.102]
39	K03821	phbC; polyhydroxyalkanoate synthase [EC:2.3.1.-]
39	K03862	vanA; vanillate monooxygenase [EC:1.14.13.82]
39	K04090	E1.2.7.8; indolepyruvate ferredoxin oxidoreductase [EC:1.2.7.8]

39	K04748	norQ; nitric oxide reductase NorQ protein
39	K04751	glnB; nitrogen regulatory protein P-II 1
39	K04761	oxyR; LysR family transcriptional regulator, hydrogen peroxide-inducible genes activator
39	K04764	ihfA; integration host factor subunit alpha
39	K04771	degP; serine protease Do [EC:3.4.21.107]
39	K05589	ftsB; cell division protein FtsB
39	K05808	yhbH; putative sigma-54 modulation protein
39	K05813	ugpB; sn-glycerol 3-phosphate transport system substrate-binding protein
39	K06041	kdsD; arabinose-5-phosphate isomerase [EC:5.3.1.13]
39	K06178	rluB; 23S rRNA pseudouridine2605 synthase [EC:5.4.99.22]
39	K06189	corC; magnesium and cobalt transporter
39	K06190	ispZ; intracellular septation protein
39	K06195	apaG; ApaG protein
39	K06204	dksA; DnaK suppressor protein
39	K06287	maf; septum formation protein
39	K06891	clpS; ATP-dependent Clp protease adaptor protein ClpS
39	K06955	uncharacterized protein
39	K06966	uncharacterized protein
39	K06991	uncharacterized protein
39	K07018	uncharacterized protein
39	K07025	putative hydrolase of the HAD superfamily
39	K07040	uncharacterized protein
39	K07042	ybeY; probable rRNA maturation factor
39	K07044	uncharacterized protein
39	K07058	membrane protein
39	K07080	uncharacterized protein
39	K07090	uncharacterized protein
39	K07107	ybgC; acyl-CoA thioester hydrolase [EC:3.1.2.-]
39	K07112	uncharacterized protein
39	K07114	yfbK; Ca-activated chloride channel homolog
39	K07119	uncharacterized protein
39	K07147	msrP; methionine sulfoxide reductase catalytic subunit [EC:1.8.-.-]
39	K07287	bamC; outer membrane protein assembly factor BamC
39	K07303	iorB; isoquinoline 1-oxidoreductase subunit beta [EC:1.3.99.16]
39	K07305	msrB; peptide-methionine (R)-S-oxide reductase [EC:1.8.4.12]
39	K07323	mlaC; phospholipid transport system substrate-binding protein
39	K07397	yhfA; putative redox protein
39	K08311	nudH; putative (di)nucleoside polyphosphate hydrolase [EC:3.6.1.-]
39	K08939	pucB; light-harvesting protein B-800-850 beta chain
39	K08998	uncharacterized protein
39	K09004	uncharacterized protein
39	K09710	ybeB; ribosome-associated protein
39	K09945	uncharacterized protein
39	K09969	aapJ; general L-amino acid transport system substrate-binding protein
39	K09970	aapQ; general L-amino acid transport system permease protein
39	K09971	aapM; general L-amino acid transport system permease protein
39	K09972	aapP; general L-amino acid transport system ATP-binding protein [EC:3.6.3.-]
39	K09983	uncharacterized protein
39	K10112	msmX; multiple sugar transport system ATP-binding protein
39	K10255	FAD6; acyl-lipid omega-6 desaturase (Delta-12 desaturase) [EC:1.14.19.23 1.14.19.45]
39	K10716	kch; voltage-gated potassium channel
39	K10823	oppF; oligopeptide transport system ATP-binding protein
39	K10941	fleQ; sigma-54 dependent transcriptional regulator, flagellar regulatory protein
39	K10943	flrC; two-component system, response regulator FlrC
39	K11473	glcF; glycolate oxidase iron-sulfur subunit
39	K11749	rseP; regulator of sigma E protease [EC:3.4.24.-]
39	K13292	lgt; phosphatidylglycerol:prolipoprotein diacylglycerol transferase [EC:2.-.-.-]
39	K15987	hppA; K(+)-stimulated pyrophosphate-energized sodium pump [EC:3.6.1.1]
39	K17222	soxA; sulfur-oxidizing protein SoxA
39	K17223	soxX; sulfur-oxidizing protein SoxX
39	K20034	dmdB; 3-(methylthio)propionyl---CoA ligase [EC:6.2.1.44]
38	K00133	asd; aspartate-semialdehyde dehydrogenase [EC:1.2.1.11]
38	K00339	nuoJ; NADH-quinone oxidoreductase subunit J [EC:1.6.5.3]
38	K00919	ispE; 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase [EC:2.7.1.148]
38	K00962	pnp; polyribonucleotide nucleotidyltransferase [EC:2.7.7.8]
38	K02275	coxB; cytochrome c oxidase subunit II [EC:1.9.3.1]
38	K03621	plsX; glycerol-3-phosphate acyltransferase PlsX [EC:2.3.1.15]
37	K02387	flgB; flagellar basal-body rod protein FlgB
36	K01937	pyrG; CTP synthase [EC:6.3.4.2]
36	K03040	rpoA; DNA-directed RNA polymerase subunit alpha [EC:2.7.7.6]
36	K03599	sspA; stringent starvation protein A
35	K02397	flgL; flagellar hook-associated protein 3 FlgL
35	K03582	recB; exodeoxyribonuclease V beta subunit [EC:3.1.11.5]

35	K10554	frcA; fructose transport system ATP-binding protein
34	K00059	fabG; 3-oxoacyl-[acyl-carrier protein] reductase [EC:1.1.1.100]
34	K00600	glyA; glycine hydroxymethyltransferase [EC:2.1.2.1]
34	K00831	serC; phosphoserine aminotransferase [EC:2.6.1.52]
34	K01092	E3.1.3.25; myo-inositol-1(or 4)-monophosphatase [EC:3.1.3.25]
34	K01687	ilvD; dihydroxy-acid dehydratase [EC:4.2.1.9]
34	K01689	ENO; enolase [EC:4.2.1.11]
34	K03072	secD; preprotein translocase subunit SecD
34	K03076	secY; preprotein translocase subunit SecY
34	K03769	ppiC; peptidyl-prolyl cis-trans isomerase C [EC:5.2.1.8]
34	K03770	ppiD; peptidyl-prolyl cis-trans isomerase D [EC:5.2.1.8]
34	K03823	pat; phosphinothricin acetyltransferase [EC:2.3.1.183]
33	K00334	nuoE; NADH-quinone oxidoreductase subunit E [EC:1.6.5.3]
33	K00940	ndk; nucleoside-diphosphate kinase [EC:2.7.4.6]
33	K01956	carA; carbamoyl-phosphate synthase small subunit [EC:6.3.5.5]
33	K01961	accC; acetyl-CoA carboxylase, biotin carboxylase subunit [EC:6.4.1.2 6.3.4.14]
33	K02109	ATPF0B; F-type H+-transporting ATPase subunit b
33	K02114	ATPF1E; F-type H+-transporting ATPase subunit epsilon
33	K02160	accB; acetyl-CoA carboxylase biotin carboxyl carrier protein
32	K00031	IDH1; isocitrate dehydrogenase [EC:1.1.1.42]
32	K00164	OGDH; 2-oxoglutarate dehydrogenase E1 component [EC:1.2.4.2]
32	K01624	FBA; fructose-bisphosphate aldolase, class II [EC:4.1.2.13]
32	K02518	infA; translation initiation factor IF-1
32	K02874	RP-L14; large subunit ribosomal protein L14
32	K02876	RP-L15; large subunit ribosomal protein L15
32	K02878	RP-L16; large subunit ribosomal protein L16
32	K02954	RP-S14; small subunit ribosomal protein S14
32	K02959	RP-S16; small subunit ribosomal protein S16
32	K03386	PRDX2_4; peroxiredoxin (alkyl hydroperoxide reductase subunit C) [EC:1.11.1.15]
32	K06142	hlpA; outer membrane protein
31	K03060	rpoZ; DNA-directed RNA polymerase subunit omega [EC:2.7.7.6]
31	K03100	lepB; signal peptidase I [EC:3.4.21.89]
31	K03628	rho; transcription termination factor Rho
31	K01923	purC; phosphoribosylaminoimidazole-succinocarboxamide synthase [EC:6.3.2.6]
31	K17226	soxY; sulfur-oxidizing protein SoxY
30	K00382	DLD; dihydrolipoamide dehydrogenase [EC:1.8.1.4]
30	K00826	E2.6.1.42; branched-chain amino acid aminotransferase [EC:2.6.1.42]
30	K01939	purA; adenylosuccinate synthase [EC:6.3.4.4]
30	K01952	purL; phosphoribosylformylglycinamidine synthase [EC:6.3.5.3]
30	K02834	rbfA; ribosome-binding factor A
30	K03531	ftsZ; cell division protein FtsZ
30	K03671	trxA; thioredoxin 1
30	K11927	rhlE; ATP-dependent RNA helicase RhlE [EC:3.6.4.13]
29	K00335	nuoF; NADH-quinone oxidoreductase subunit F [EC:1.6.5.3]
29	K00337	nuoH; NADH-quinone oxidoreductase subunit H [EC:1.6.5.3]
29	K00384	trxB; thioredoxin reductase (NADPH) [EC:1.8.1.9]
29	K00548	metH; 5-methyltetrahydrofolate--homocysteine methyltransferase [EC:2.1.1.13]
29	K00873	PK; pyruvate kinase [EC:2.7.1.40]
29	K01807	rpiA; ribose 5-phosphate isomerase A [EC:5.3.1.6]
29	K01955	carB; carbamoyl-phosphate synthase large subunit [EC:6.3.5.5]
29	K02314	dnaB; replicative DNA helicase [EC:3.6.4.12]
29	K02836	prfB; peptide chain release factor 2
29	K02988	RP-S5; small subunit ribosomal protein S5
29	K05896	scpA; segregation and condensation protein A
28	K00336	nuoG; NADH-quinone oxidoreductase subunit G [EC:1.6.5.3]
28	K00526	E1.17.4.1B; ribonucleoside-diphosphate reductase beta chain [EC:1.17.4.1]
28	K02888	RP-L21; large subunit ribosomal protein L21
28	K02897	RP-L25; large subunit ribosomal protein L25
28	K02968	RP-S20; small subunit ribosomal protein S20
28	K03086	SIG1; RNA polymerase primary sigma factor
28	K03569	mreB; rod shape-determining protein MreB and related proteins
27	K01652	E2.2.1.6L; acetolactate synthase I/II/III large subunit [EC:2.2.1.6]
27	K02416	fliM; flagellar motor switch protein FliM
27	K02890	RP-L22; large subunit ribosomal protein L22
27	K01876	DARS; aspartyl-tRNA synthetase [EC:6.1.1.12]
26	K00208	fabI; enoyl-[acyl-carrier protein] reductase I [EC:1.3.1.9 1.3.1.10]
26	K00343	nuoN; NADH-quinone oxidoreductase subunit N [EC:1.6.5.3]
26	K00793	ribE; riboflavin synthase [EC:2.5.1.9]
26	K01167	E3.1.27.3; ribonuclease T1 [EC:3.1.27.3]
26	K01868	TARS; threonyl-tRNA synthetase [EC:6.1.1.3]
26	K02112	ATPF1B; F-type H+-transporting ATPase subunit beta [EC:3.6.3.14]
26	K03798	ftsH; cell division protease FtsH [EC:3.4.24.-]
26	K09903	pyrH; uridylate kinase [EC:2.7.4.22]

25	K00928	lysC; aspartate kinase [EC:2.7.2.4]
25	K02970	RP-S21; small subunit ribosomal protein S21
25	K03544	clpX; ATP-dependent Clp protease ATP-binding subunit ClpX
25	K00163	aceE; pyruvate dehydrogenase E1 component [EC:1.2.4.1]
24	K00053	ilvC; ketol-acid reductoisomerase [EC:1.1.1.86]
24	K00574	cfa; cyclopropane-fatty-acyl-phospholipid synthase [EC:2.1.1.79]
24	K01649	leuA; 2-isopropylmalate synthase [EC:2.3.3.13]
24	K03210	yajC; preprotein translocase subunit YajC
24	K13628	iscA; iron-sulfur cluster assembly protein
23	K00331	nuoB; NADH-quinone oxidoreductase subunit B [EC:1.6.5.3]
23	K01733	thrC; threonine synthase [EC:4.2.3.1]
23	K01999	livK; branched-chain amino acid transport system substrate-binding protein
23	K02276	coxC; cytochrome c oxidase subunit III [EC:1.9.3.1]
23	K03075	secG; preprotein translocase subunit SecG
23	K03116	tatA; sec-independent protein translocase protein TatA
23	K03676	grxC; glutaredoxin 3
22	K03071	secB; preprotein translocase subunit SecB
22	K03089	SIG3.3.1; RNA polymerase sigma-32 factor
22	K03536	rnpA; ribonuclease P protein component [EC:3.1.26.5]
22	K03666	hfq; host factor-I protein
22	K00241	sdhC; succinate dehydrogenase / fumarate reductase, cytochrome b subunit
22	K03609	minD; septum site-determining protein MinD
22	K03641	tolB; TolB protein
22	K07277	SAM50; outer membrane protein insertion porin family
21	K01902	sucD; succinyl-CoA synthetase alpha subunit [EC:6.2.1.5]
21	K02338	dnaN; DNA polymerase III subunit beta [EC:2.7.7.7]
21	K02355	fusA; elongation factor G
20	K02884	RP-L19; large subunit ribosomal protein L19
20	K02990	RP-S6; small subunit ribosomal protein S6
20	K03073	secE; preprotein translocase subunit SecE
19	K02909	RP-L31; large subunit ribosomal protein L31
19	K02913	RP-L33; large subunit ribosomal protein L33
19	K02933	RP-L6; large subunit ribosomal protein L6
19	K02967	RP-S2; small subunit ribosomal protein S2
19	K02982	RP-S3; small subunit ribosomal protein S3
18	K03799	htpX; heat shock protein HtpX [EC:3.4.24.-]
18	K00239	sdhA; succinate dehydrogenase / fumarate reductase, flavoprotein subunit [EC:1.3.5.1 1.3.5.4]
18	K02881	RP-L18; large subunit ribosomal protein L18
18	K02899	RP-L27; large subunit ribosomal protein L27
18	K02904	RP-L29; large subunit ribosomal protein L29
18	K02946	RP-S10; small subunit ribosomal protein S10
18	K03117	tatB; sec-independent protein translocase protein TatB
18	K03665	hflX; GTPase
18	K03687	GRPE; molecular chaperone GrpE
18	K03746	hns; DNA-binding protein H-NS
18	K07516	fadN; 3-hydroxyacyl-CoA dehydrogenase [EC:1.1.1.35]
18	K07735	algH; putative transcriptional regulator
18	K09159	cptB; antitoxin CptB
17	K02405	fliA; RNA polymerase sigma factor for flagellar operon FliA
17	K01704	leuD; 3-isopropylmalate/(R)-2-methylmalate dehydratase small subunit [EC:4.2.1.33 4.2.1.35]
17	K02389	flgD; flagellar basal-body rod modification protein FlgD
17	K02391	flgF; flagellar basal-body rod protein FlgF
17	K02412	fliI; flagellum-specific ATP synthase [EC:3.6.3.14]
17	K02420	fliQ; flagellar biosynthetic protein FliQ
17	K02527	kdtA; 3-deoxy-D-manno-octulosonic-acid transferase [EC:2.4.99.12 2.4.99.13 2.4.99.14 2.4.99.15]
17	K04061	flhB2; flagellar biosynthesis protein
17	K08926	pufA; light-harvesting complex 1 alpha chain
16	K01682	acnB; aconitate hydratase 2 / 2-methylisocitrate dehydratase [EC:4.2.1.3 4.2.1.99]
16	K01696	trpB; tryptophan synthase beta chain [EC:4.2.1.20]
16	K01889	FARSA; phenylalanyl-tRNA synthetase alpha chain [EC:6.1.1.20]
16	K01915	glnA; glutamine synthetase [EC:6.3.1.2]
16	K02116	atpI; ATP synthase protein I
16	K04488	iscU; nitrogen fixation protein NifU and related proteins
15	K03545	tig; trigger factor
15	K02388	flgC; flagellar basal-body rod protein FlgC
15	K02394	flgI; flagellar P-ring protein precursor FlgI
15	K04562	flhG; flagellar biosynthesis protein FlhG
14	K03694	clpA; ATP-dependent Clp protease ATP-binding subunit ClpA
14	K02417	fliNY; flagellar motor switch protein FliN/FliY
14	K02886	RP-L2; large subunit ribosomal protein L2
14	K02926	RP-L4; large subunit ribosomal protein L4
14	K02931	RP-L5; large subunit ribosomal protein L5
14	K02965	RP-S19; small subunit ribosomal protein S19

13	K00024	mdh; malate dehydrogenase [EC:1.1.1.37]
13	K00658	DLST; 2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase) [EC:2.3.1.61]
13	K01647	CS; citrate synthase [EC:2.3.3.1]
13	K02895	RP-L24; large subunit ribosomal protein L24
13	K02939	RP-L9; large subunit ribosomal protein L9
13	K02945	RP-S1; small subunit ribosomal protein S1
12	K01507	ppa; inorganic pyrophosphatase [EC:3.6.1.1]
12	K02415	fliL; flagellar FliL protein
12	K03286	TC.OOP; OmpA-OmpF porin, OOP family
12	K04077	groEL; chaperonin GroEL
12	K04564	SOD2; superoxide dismutase, Fe-Mn family [EC:1.15.1.1]
12	K15724	erpA; iron-sulfur cluster insertion protein
12	K03640	pal; peptidoglycan-associated lipoprotein
12	K03925	mraZ; MraZ protein
12	K19416	yccA; modulator of FtsH protease
11	K01494	dcd; dCTP deaminase [EC:3.5.4.13]
11	K01817	trpF; phosphoribosylanthranilate isomerase [EC:5.3.1.24]
11	K01892	HARS; histidyl-tRNA synthetase [EC:6.1.1.21]
11	K02406	fliC; flagellin
11	K02556	motA; chemotaxis protein MotA
11	K03979	obgE; GTPase [EC:3.6.5.-]
11	K09001	anmK; anhydro-N-acetylmuramic acid kinase [EC:2.7.1.170]
11	K14652	ribBA; 3,4-dihydroxy 2-butanone 4-phosphate synthase / GTP cyclohydrolase II [EC:4.1.99.12 3.5.4.25]
10	K00411	UQCRFS1; ubiquinol-cytochrome c reductase iron-sulfur subunit [EC:1.10.2.2]
10	K00124	fdoH; formate dehydrogenase iron-sulfur subunit
10	K00525	E1.17.4.1A; ribonucleoside-diphosphate reductase alpha chain [EC:1.17.4.1]
10	K02301	cyoE;
10	K04087	hflC; membrane protease subunit HflC [EC:3.4.-.-]
10	K04088	hflK; membrane protease subunit HflK [EC:3.4.-.-]
10	K17227	soxZ; sulfur-oxidizing protein SoxZ
9	K00036	G6PD; glucose-6-phosphate 1-dehydrogenase [EC:1.1.1.49 1.1.1.363]
9	K02115	ATPF1G; F-type H+-transporting ATPase subunit gamma
9	K02356	efp; elongation factor P
9	K02601	nusG; transcriptional antiterminator NusG
9	K02879	RP-L17; large subunit ribosomal protein L17
9	K02916	RP-L35; large subunit ribosomal protein L35
9	K03043	rpoB; DNA-directed RNA polymerase subunit beta [EC:2.7.7.6]
8	K00432	gpx; glutathione peroxidase [EC:1.11.1.9]
8	K02040	pstS; phosphate transport system substrate-binding protein
7	K00338	nuoI; NADH-quinone oxidoreductase subunit I [EC:1.6.5.3]
7	K00412	CYTB; ubiquinol-cytochrome c reductase cytochrome b subunit
7	K02078	acpP; acyl carrier protein
7	K02860	rimM; 16S rRNA processing protein RimM
7	K02911	RP-L32; large subunit ribosomal protein L32
7	K03521	fixA; electron transfer flavoprotein beta subunit
7	K03522	fixB; electron transfer flavoprotein alpha subunit
7	K03530	hupB; DNA-binding protein HU-beta
7	K04487	iscS; cysteine desulfurase [EC:2.8.1.7]
7	K09458	fabF; 3-oxoacyl-[acyl-carrier-protein] synthase II [EC:2.3.1.179]
6	K02600	nusA; N utilization substance protein A
5	K00330	nuoA; NADH-quinone oxidoreductase subunit A [EC:1.6.5.3]
5	K00413	CYC1; ubiquinol-cytochrome c reductase cytochrome c1 subunit
5	K00626	E2.3.1.9; acetyl-CoA C-acetyltransferase [EC:2.3.1.9]
5	K00674	dapD; 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase [EC:2.3.1.117]
5	K02469	gyrA; DNA gyrase subunit A [EC:5.99.1.3]
5	K03564	BCP; peroxiredoxin Q/BCP [EC:1.11.1.15]
5	K12340	tolC; outer membrane protein
4	K00332	nuoC; NADH-quinone oxidoreductase subunit C [EC:1.6.5.3]
4	K00342	nuoM; NADH-quinone oxidoreductase subunit M [EC:1.6.5.3]
4	K01358	clpP; ATP-dependent Clp protease, protease subunit [EC:3.4.21.92]
4	K02036	pstB; phosphate transport system ATP-binding protein [EC:3.6.3.27]
4	K02039	phoU; phosphate transport system protein
4	K02519	infB; translation initiation factor IF-2
4	K03406	mcp; methyl-accepting chemotaxis protein
3	K03387	ahpF; alkyl hydroperoxide reductase subunit F [EC:1.6.4.-]
3	K00242	sdhD; succinate dehydrogenase / fumarate reductase, membrane anchor subunit
3	K00948	PRPS; ribose-phosphate pyrophosphokinase [EC:2.7.6.1]
3	K02014	TC.FEV.OM; iron complex outermembrane recepter protein
3	K02051	ABC.SN.S; NitT/TauT family transport system substrate-binding protein
3	K05524	fdxA; ferredoxin
3	K08738	CYC; cytochrome c
2	K00003	E1.1.1.3; homoserine dehydrogenase [EC:1.1.1.3]
2	K00012	UGDH; UDPglucose 6-dehydrogenase [EC:1.1.1.22]

2 K00052 leuB; 3-isopropylmalate dehydrogenase [EC:1.1.1.85]
2 K00057 gpsA; glycerol-3-phosphate dehydrogenase (NAD(P)+) [EC:1.1.1.94]
2 K00134 GAPDH; glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12]
2 K00210.K00800 E1.3.1.12; prephenate dehydrogenase [EC:1.3.1.12]
2 K00333 nuoD; NADH-quinone oxidoreductase subunit D [EC:1.6.5.3]
2 K00616 E2.2.1.2; transaldolase [EC:2.2.1.2]
2 K00620 argJ; glutamate N-acetyltransferase / amino-acid N-acetyltransferase [EC:2.3.1.35 2.3.1.1]
2 K00627 DLAT; pyruvate dehydrogenase E2 component (dihydrolipoamide acetyltransferase) [EC:2.3.1.12]
2 K00790 murA; UDP-N-acetylglucosamine 1-carboxyvinyltransferase [EC:2.5.1.7]
2 K00794 ribH; 6,7-dimethyl-8-ribityllumazine synthase [EC:2.5.1.78]
2 K00800 aroA; 3-phosphoshikimate 1-carboxyvinyltransferase [EC:2.5.1.19]
2 K00832 tyrB; aromatic-amino-acid transaminase [EC:2.6.1.57]
2 K00845 glk; glucokinase [EC:2.7.1.2]
2 K00937 ppk; polyphosphate kinase [EC:2.7.4.1]
2 K00990 glnD; [protein-PII] uridylyltransferase [EC:2.7.7.59]
2 K01029 E2.8.3.5B; 3-oxoacid CoA-transferase subunit B [EC:2.8.3.5]
2 K01251 E3.3.1.1; adenosylhomocysteinase [EC:3.3.1.1]
2 K01256 pepN; aminopeptidase N [EC:3.4.11.2]
2 K01265 map; methionyl aminopeptidase [EC:3.4.11.18]
2 K01414 prlC; oligopeptidase A [EC:3.4.24.70]
2 K01596 E4.1.1.32; phosphoenolpyruvate carboxykinase (GTP) [EC:4.1.1.32]
2 K01674 cah; carbonic anhydrase [EC:4.2.1.1]
2 K01810 GPI; glucose-6-phosphate isomerase [EC:5.3.1.9]
2 K01870 IARS; isoleucyl-tRNA synthetase [EC:6.1.1.5]
2 K01872 AARS; alanyl-tRNA synthetase [EC:6.1.1.7]
2 K01873 VARS; valyl-tRNA synthetase [EC:6.1.1.9]
2 K01879 glyS; glycyl-tRNA synthetase beta chain [EC:6.1.1.14]
2 K01933 purM; phosphoribosylformylglycinamidine cyclo-ligase [EC:6.3.3.1]
2 K01940 argG; argininosuccinate synthase [EC:6.3.4.5]
2 K01990 ABC-2.A; ABC-2 type transport system ATP-binding protein
2 K01992 ABC-2.P; ABC-2 type transport system permease protein
2 K02037 pstC; phosphate transport system permease protein
2 K02108 ATPF0A; F-type H+-transporting ATPase subunit a
2 K02110 ATPF0C; F-type H+-transporting ATPase subunit c
2 K02113 ATPF1D; F-type H+-transporting ATPase subunit delta
2 K02434 gatB; aspartyl-tRNA(Asn)/glutamyl-tRNA(Gln) amidotransferase subunit B [EC:6.3.5.6 6.3.5.7]
2 K02575 NRT; MFS transporter, NNP family, nitrate/nitrite transporter
2 K02686 priB; primosomal replication protein N
2 K02687 prmA; ribosomal protein L11 methyltransferase [EC:2.1.1.-]
2 K02945.K03527 RP-S1; small subunit ribosomal protein S1
2 K03177 truB; tRNA pseudouridine55 synthase [EC:5.4.99.25]
2 K03183 ubiE; demethylmenaquinone methyltransferase / 2-methoxy-6-polyprenyl-1,4-benzoquinol methylase
2 K03559 exbD; biopolymer transport protein ExbD
2 K03561 exbB; biopolymer transport protein ExbB
2 K03595 era; GTPase
2 K03624 greA; transcription elongation factor GreA
2 K03625 nusB; N utilization substance protein B
2 K03664 smpB; SsrA-binding protein
2 K03701 uvrA; excinuclease ABC subunit A
2 K03705 hrcA; heat-inducible transcriptional repressor
2 K03811 pnuC; nicotinamide mononucleotide transporter
2 K04078 groES; chaperonin GroES
2 K04082 hscB; molecular chaperone HscB
2 K04567 KARS; lysyl-tRNA synthetase, class II [EC:6.1.1.6]
2 K05794 terC; tellurite resistance protein TerC
2 K05973 phaZ; poly(3-hydroxybutyrate) depolymerase [EC:3.1.1.75]
2 K06024 scpB; segregation and condensation protein B
2 K06147 ABCB-BAC; ATP-binding cassette, subfamily B, bacterial
2 K06194 nlpD; lipoprotein NlpD
2 K06442 tlyA; 23S rRNA (cytidine1920-2'-O)/16S rRNA (cytidine1409-2'-O)-methyltransferase [EC:2.1.1.226 2.1.1.227]
2 K06942 ychF; ribosome-binding ATPase
2 K07056 rsmI; 16S rRNA (cytidine1402-2'-O)-methyltransferase [EC:2.1.1.198]
2 K07146 UPF0176 protein
2 K07636 phoR; two-component system, OmpR family, phosphate regulon sensor histidine kinase PhoR [EC:2.7.13.3]
2 K07657 phoB; two-component system, OmpR family, phosphate regulon response regulator PhoB
2 K08300 rne; ribonuclease E [EC:3.1.26.12]
2 K08930 pucA; light-harvesting protein B-800-850 alpha chain
2 K09117 uncharacterized protein
2 K09861 uncharacterized protein
2 K14441 rimO; ribosomal protein S12 methylthiotransferase [EC:2.8.4.4]
2 K17103 CHO1; CDP-diacylglycerol---serine O-phosphatidyltransferase [EC:2.7.8.8]
1 K02398 flgM; negative regulator of flagellin synthesis FlgM
1 K09860 uncharacterized protein

Supplementary 6: List of functions were significantly more expressed in B30 sample.

NADH:quinone oxidoreductase, prokaryotes
Thiosulfate oxidation by SOX complex, thiosulfate => sulfate
Reductive citrate cycle (Arnon-Buchanan cycle)
Sec (secretion) system
Dissimilatory sulfate reduction, sulfate => H2S
Citrate cycle (TCA cycle, Krebs cycle)
Complete nitrification, comammox, ammonia => nitrite => nitrate
Citrate cycle, second carbon oxidation, 2-oxoglutarate => oxaloacetate
Riboflavin biosynthesis, GTP => riboflavin/FMN/FAD
Dicarboxylate-hydroxybutyrate cycle
Photorespiration
Fatty acid biosynthesis, elongation
Succinate dehydrogenase, prokaryotes
Methane oxidation, methanotroph, methane => formaldehyde
3-Hydroxypropionate bi-cycle
Cytochrome bc1 complex
Nitrification, ammonia => nitrite
Cytochrome bc1 complex respiratory unit
Glycolysis (Embden-Meyerhof pathway), glucose => pyruvate
Tetrahydrofolate biosynthesis, GTP => THF
RNA polymerase, bacteria
Putative ABC transport system
C1-unit interconversion, prokaryotes
Gluconeogenesis, oxaloacetate => fructose-6P
Glycolysis, core module involving three-carbon compounds
Phosphate transport system

Supplementary 7: Metabolic changes of Comamonadaceae members from freshwater (NER) to saline water (B30). List of functions that are unique for each of the samples, NER and B30 samples, respectively.

	NER	B30
ko02000 Transporters	ko:K02037 pstC; phosphate transport system permease protein ko:K01992 ABC-2.P; ABC-2 type transport system permease protein ko:K01990 ABC-2.A; ABC-2 type transport system ATP-binding protein ko:K02575 NRT; MFS transporter, NNP family, nitrate/nitrite transporter ko:K03561 exbB; biopolymer transport protein ExbB ko:K03559 exbD; biopolymer transport protein ExbD ko:K03562 tolQ; biopolymer transport protein TolQ ko:K04044 hscA; molecular chaperone HscA ko:K03811 pnuC; nicotinamide mononucleotide transporter	ko:K02012 afuA; iron(III) transport system substrate-binding protein ko:K02011 afuB; iron(III) transport system permease protein ko:K02010 afuC; iron(III) transport system ATP-binding protein ko:K02025 ABC.MS.P; multiple sugar transport system permease protein ko:K01997 livH; branched-chain amino acid transport system permease protein ko:K01998 livM; branched-chain amino acid transport system permease protein ko:K01995 livG; branched-chain amino acid transport system ATP-binding protein ko:K01996 livF; branched-chain amino acid transport system ATP-binding protein ko:K02003 ABC.CD.A; putative ABC transport system ATP-binding protein ko:K02014 Iron complex outermembrane recepter protein ko:K02051 ABC.SN.S; NitT/TauT family transport system substrate-binding protein ko:K02050 ABC.SN.P; NitT/TauT family transport system permease protein ko:K02067 mlaD; phospholipid/cholesterol/gamma-HCH transport system substrate-binding protein ko:K02065 mlaF; phospholipid/cholesterol/gamma-HCH transport system ATP-binding protein ko:K02027 ABC.MS.S; multiple sugar transport system substrate-binding protein ko:K02026 ABC.MS.P1; multiple sugar transport system permease protein ko:K02058 ABC.SS.S; simple sugar transport system substrate-binding protein ko:K02044 phnD; phosphonate transport system substrate-binding protein ko:K02041 phnC; phosphonate transport system ATP-binding protein ko:K02035 ABC.PE.S; peptide/nickel transport system substrate-binding protein ko:K02033 ABC.PE.P; peptide/nickel transport system permease protein ko:K02034 ABC.PE.P1; peptide/nickel transport system permease protein ko:K02031 ABC.PE.A; peptide/nickel transport system ATP-binding protein ko:K02032 ABC.PE.A1; peptide/nickel transport system ATP-binding protein ko:K02821 PTS-Ula-EIIA; PTS system, ascorbate-specific IIA component ko:K03284 corA; magnesium transporter ko:K03549 kup; KUP system potassium uptake protein ko:K03640 pal; peptidoglycan-associated lipoprotein ko:K03585 acrA; membrane fusion protein, multidrug efflux system ko:K02198 ccmF; cytochrome c-type biogenesis protein CcmF ko:K10112 msmX; multiple sugar transport system ATP-binding protein ko:K05813 ugpB; sn-glycerol 3-phosphate transport system substrate-binding protein ko:K05814 ugpA; sn-glycerol 3-phosphate transport system permease protein ko:K05815 ugpE; sn-glycerol 3-phosphate transport system permease protein ko:K07323 mlaC; phospholipid transport system substrate-binding protein ko:K07122 mlaB; phospholipid transport system transporter-binding protein ko:K09970 aapQ; general L-amino acid transport system permease protein ko:K09971 aapM; general L-amino acid transport system permease protein ko:K09972 aapP; general L-amino acid transport system ATP-binding protein ko:K10823 oppF; oligopeptide transport system ATP-binding protein ko:K11189 PTS-HPR; phosphocarrier protein ko:K07114 yfbK; Ca-activated chloride channel homolog ko:K06076 fadL; long-chain fatty acid transport protein ko:K07277 SAM50; outer membrane protein insertion porin family ko:K07287 bamC; outer membrane protein assembly factor BamC ko:K06186 bamE; outer membrane protein assembly factor BamE ko:K03980 murJ; putative peptidoglycan lipid II flippase ko:K06189 corC; magnesium and cobalt transporter ko:K19416 yccA; modulator of FtsH protease ko:K18138 acrB; multidrug efflux pump
ko01007 Amino acid related enzymes	ko:K01870 IARS; isoleucyl-tRNA synthetase ko:K01873 VARS; valyl-tRNA synthetase ko:K04567 KARS; lysyl-tRNA synthetase, class II ko:K01879 glyS; glycyl-tRNA synthetase beta chain ko:K01868 TARS; threonyl-tRNA synthetase ko:K01872 AARS; alanyl-tRNA synthetase ko:K01889 FARSA; phenylalanyl-tRNA synthetase alpha chain ko:K00832 tyrB; aromatic-amino-acid transaminase	ko:K01894 gluQ; glutamyl-Q tRNA(Asp) synthetase ko:K01866 YARS; tyrosyl-tRNA synthetase ko:K01883 CARS; cysteinyl-tRNA synthetase ko:K01876 DARS; aspartyl-tRNA synthetase ko:K01878 glyQ; glycyl-tRNA synthetase alpha chain ko:K01875 SARS; seryl-tRNA synthetase ko:K01890 FARSB; phenylalanyl-tRNA synthetase beta chain ko:K00822 E2.6.1.18; beta-alanine--pyruvate transaminase ko:K01845 hemL; glutamate-1-semialdehyde 2,1-aminomutase ko:K03430 phnW; 2-aminoethylphosphonate-pyruvate transaminase ko:K14287 ybdL; methionine transaminase

	NER	B30
ko02035 Bacterial motility proteins	ko:K03407 cheA; two-component system, chemotaxis family, sensor kinase CheA ko:K02391 flgF; flagellar basal-body rod protein FlgF ko:K02414 fliK; flagellar hook-length control protein FliK	ko:K00575 cheR; chemotaxis protein methyltransferase CheR ko:K03408 cheW; purine-binding chemotaxis protein CheW ko:K03412 cheB; two-component system, chemotaxis family, response regulator CheB ko:K02412 fliI; flagellum-specific ATP synthase ko:K02419 fliP; flagellar biosynthetic protein FliP ko:K02420 fliQ; flagellar biosynthetic protein FliQ ko:K02410 fliG; flagellar motor switch protein FliG ko:K02417 fliNY; flagellar motor switch protein FliN/FliY ko:K02409 fliF; flagellar M-ring protein FliF ko:K02394 flgI; flagellar P-ring protein precursor FlgI ko:K02393 flgH; flagellar L-ring protein precursor FlgH ko:K02388 flgC; flagellar basal-body rod protein FlgC ko:K02392 flgG; flagellar basal-body rod protein FlgG ko:K02395 flgJ; flagellar protein FlgJ ko:K02396 flgK; flagellar hook-associated protein 1 FlgK ko:K02397 flgL; flagellar hook-associated protein 3 FlgL ko:K02407 fliD; flagellar hook-associated protein 2 ko:K02405 fliA; RNA polymerase sigma factor for flagellar operon FliA ko:K02404 flhF; flagellar biosynthesis protein FlhF ko:K02650 pilA; type IV pilus assembly protein PilA
ko01002 Peptidases	ko:K01256 pepN; aminopeptidase N ko:K01265 map; methionyl aminopeptidase ko:K03798 ftsH; cell division protease FtsH	ko:K00764 purF; amidophosphoribosyltransferase ko:K01255 CARP; leucyl aminopeptidase ko:K01451 hipO; hippurate hydrolase ko:K01356 lexA; repressor LexA ko:K01297 ldcA; muramoyltetrapeptide carboxypeptidase ko:K00681 ggt; gamma-glutamyltranspeptidase / glutathione hydrolase ko:K03797 E3.4.21.102; carboxyl-terminal processing protease ko:K11749 rseP; regulator of sigma E protease ko:K04771 degP; serine protease Do ko:K07262 pbpG; serine-type D-Ala-D-Ala endopeptidase (penicillin-binding protein 7)
ko02044 Secretion system	ko:K03073 secE; preprotein translocase subunit SecE	ko:K02452 gspC; general secretion pathway protein C ko:K02453 gspD; general secretion pathway protein D ko:K02456 gspG; general secretion pathway protein G ko:K02650 pilA; type IV pilus assembly protein PilA ko:K02409 fliF; flagellar M-ring protein FliF ko:K02412 fliI; flagellum-specific ATP synthase ko:K02417 fliNY; flagellar motor switch protein FliN/FliY ko:K02419 fliP; flagellar biosynthetic protein FliP ko:K02420 fliQ; flagellar biosynthetic protein FliQ ko:K03070 secA; preprotein translocase subunit SecA ko:K03074 secF; preprotein translocase subunit SecF ko:K03117 tatB; sec-independent protein translocase protein TatB ko:K03118 tatC; sec-independent protein translocase protein TatC ko:K04061 flhB2; flagellar biosynthesis protein

Discusión general

Los estuarios son uno de los ecosistemas acuáticos más dinámicos y ricos en diversidad de especies del mundo [1]. Debido a los cambios de las corrientes producidas por la descarga de los ríos y los ciclos mareales, los estuarios tienen una gran variabilidad físico-química interna, lo que provoca que las comunidades presentes estén constantemente adaptándose a estos cambios o moviéndose con la masa de agua en la que se encuentren mejor adaptados [2–5]. Por estas razones los estuarios son lugares donde es posible observar los cambios adaptativos del metabolismo de las diferentes especies acuáticas que están presentes en ellos [6]. Así, en este trabajo de tesis nos hemos planteado el objetivo de conocer la dinámica de las comunidades bacterianas en este ecosistema, y desentrañar qué factores ambientales afectan a la distribución y metabolismo de estos organismos. Para ello, en la presente investigación nos hemos centrado en los estuarios de Bilbao y Urdaibai: El primero de alto impacto antropogénico y el segundo una reserva de la biosfera. De esta manera hemos podido caracterizar y comparar las dinámicas anuales de las comunidades bacterianas en ambos estuarios, y al mismo tiempo, determinar cuán diferentes son. Estos estuarios, aparte de diferir un su impacto antropogénico, difieren en su estructura orográfica lo cual afecta la dinámica hidrológica del estuario, y por ende, sobre las comunidades planctónicas.

Los efectos medioambientales sobre la comunidad microbiana de los estuarios

En ambos estuarios se ha observado que los factores medio-ambientales con mayor peso en la determinación de la comunidad bacteriana, aparte de la salinidad, son la temperatura y la descarga del rio. Lo hemos observado tanto al comparar las comunidades de verano e invierno,

similar a lo indicado en los trabajos realizados por Jeffries y colaboradores (2016) [7], como al comparar los periodos de altas y bajas precipitaciones, también observado en estuario de Aveiro por Almeida y colaboradores (2006) [8]. Por un lado, la temperatura afecta a las bacterias más termosensibles de forma que éstas reducen su abundancia cuando la temperatura no es la más óptima para su desarrollo [9–12]. Así, encontramos diversas bacterias con abundancia superior en verano (familias *Pseudomonadaceae* y *Sphingobacteriaceae*), mientras que otras son significativamente más abundantes en invierno (familias *Pseudomonadaceae* y *Sphingobacteriaceae*) (capítulos I y II). El seguimiento de estas bacterias a largo plazo puede ser de gran interés y utilidad, ya que las oscilaciones de sus abundancias podrían llegar a ser correlacionadas con cambios climatológicos, como los derivados del calentamiento global [13]. Por otro lado, las precipitaciones alteran en gran medida las comunidades en los estuarios [8]. En los periodos de altas precipitaciones, los ríos aumentan su descarga y con ello las masas de agua salinas y salobres se desplazan hacia el océano [14]. El desplazamiento de las aguas conlleva también el desplazamiento de sus comunidades, por lo que las comunidades de agua dulce se vuelven más abundantes en el estuario en los periodos de altas precipitaciones, si lo comparamos con periodos más secos. Este cambio es más significativo en las aguas salobres, en donde las comunidades de agua dulce y salada se mezclan en base a la intensidad de la marea, la descarga de los tributarios y la propia estructura del estuario [15]. Es por ello que las variaciones observadas en estas masas de agua son específicas para cada estuario, y son dependientes de las variaciones debidas a las estaciones de año [16]. Así, el grado de variabilidad observado en las aguas salobres de diferentes estuarios y en diferentes estaciones es amplio. A pesar de ello existe al menos una característica que se mantiene constante en todos ellos: las aguas salobres muestran los valores de riqueza microbiana (alpha diversidad) más altos, dado que en ellas conviven tanto las bacterias de agua dulce como las de agua salada [17]. Por esta razón sería interesante su estudio, ya que en dichas aguas el metabolismo de ambas comunidades, la de agua dulce y la salina, se ve alterado para hacer frente al cambio ambiental [18]. El estudio de dicha alteración sería óptimo en estuarios con un gradiente de salinidad con la suficiente entidad

como para contener una comunidad de bacterias de una salinidad propia y estable en el tiempo; sin embargo esta característica no se da en ninguno de los dos estuarios estudiados en esta investigación. Por esta razón, pese a encontrar en varias muestras la mezcla de aguas, las comunidades encontradas son muy esporádicas mostrando gran variabilidad en el tiempo. En el caso del estuario de Bilbao, las aguas de mezcla se detectan en la superficie del último tramo del estuario en el periodo de mayores precipitaciones, pero apenas tiene unas horas de existencia debido a la velocidad del agua y a la corta longitud del estuario (capitulo II). Debido a esta gran variabilidad temporal y la escasa entidad de estas masas de agua, en esta tesis no se ha realizado un estudio más exhaustivo de las comunidades de las aguas salobres en los estuarios de Bilbao y Urdaibai.

En lo referente al impacto de la orografía en la dinámica de aguas y en la dinámica de las comunidades bacterianas, ambos estuarios se han observado características diferentes,. El estuario de Urdaibai está mezclado verticalmente, tiene lecho arenoso y con cada ciclo mareal se vacía por completo y se vuelve a llenar [19–23].Por el contrario, el Estuario de Bilbao tiene una columna de agua muy estratificada en el interior, tiene lecho fangoso, no se vacía con cada ciclo mareal y está completamente canalizado [14,24]. Estas características hacen que las comunidades bacterianas de cada estuario funcionen de forma diferente y existan diferencias sustanciales en su dinámica. Así, en Urdaibai, al tener un lecho arenoso, cada ciclo mareal da lugar a un proceso de resuspensión del material existente entre los granos de arena [22,25], y con ello también se resuspenden en el agua los microorganismos que están presentes en dicho material. Ésta puede ser la razón que explique el por qué en Urdaibai, pese a que su comunidad mayoritaria sea oceánica-costera (capítulo I), se observa una comunidad tanto fitoplanctónica [22] como bacteriana diferente en el interior del estuario respecto al exterior. Este resultado es relevante, ya que en los estuarios de drenaje, como es este caso, apenas se realizan estudios de este tipo asumiendo que las comunidades presentes en dichos ecosistemas están compuestos únicamente por comunidades oceánico-costeras. Por otro lado, en el estuario de Bilbao también

se observa diferenciación entre las comunidades de agua euhalinas del interior respecto a las más costeras. Sin embargo, en este caso el efecto no se debe tanto a la resuspensión provocada por la marea, ya que el estuario no se llega a vaciar por complete, sino a las corrientes acuáticas internas del estuario [26]. Al estar completamente canalizado [27], la tasa de intercambio entre las aguas euhalinas en el interior del estuario es significativamente menor al intercambio que muestran las masas más costeras [26]. De este modo el agua euhalina del interior del estuario (B30) apenas interacciona con el resto de las aguas euhalinas mas exteriores (B33 y B35). Por lo tanto las características físico-químicas y las comunidades microbianas del agua euhalina del interior (B30) son significativamente diferentes a las del resto de aguas euhalinas a lo largo de todo el año [14], como se ha descrito en el capítulo II. Además, al hecho de que la masa de agua interior (B30) se encuentre siempre dentro del canal, aislándose en gran medida del resto de aguas oceánicas, hay que añadir que durante la mayor parte del año se encuentra cubierta bajo una capa de agua dulce. Así, en el interior del estuario se da una estratificación vertical de salinidad, o haloclina, que impide el intercambio efectivo de compuestos desde las capas inferiores hasta las superiores [26]. Como resultado, la masa de agua euhalina del interior del estuario se convierte en un medio anóxico en verano tras el *bloom* de los organismos fotosintéticos, que quedan atrapados en la misma y se descomponen originando una reducción drástica de la concentración de oxígeno [14,26,28,29]. Estas características ambientales propias provocan que las comunidades bacterianas euhalinas del interior se diferencien del resto de aguas salinas del estuario (capitulo II), las cuales presentan mayores abundancias de las bacterias pertenecientes a los grupos *Comamonadaceae* y *Flavobacterium* y con menor abundancias de *Halomonadaceae*, *Piscirickettsiaceae* y *Pelagibacteraceae*, las cuales son más abundantes en el exterior.

Impacto antrópico en la actividad metabólica bacteriana del estuario de Bilbao

Con el fin de analizar el impacto metabólico ejercido en la comunidad microbiana por distintas causas antrópicas (p. ej. contaminación debida a actividad industrial [30], canalización [26] y vertidos del EDAR) hemos realizado un análisis metagenómico y metatranscriptómico de las diferentes masas de agua en el estuario de Bilbao centrándonos en las masas de agua más características como describimos en el capítulo II: el agua dulce (NER), la masa de agua eutrofizada del interior (B30) y la masa de agua más externa del estuario (B35).

La gran mayoría de los estudios sobre estuarios se han centrado en estudiar el impacto provocado por los vertidos contaminantes. En el caso del estuario de Bilbao, la mayor parte de los vertidos contaminantes han procedido de la industria pesada que estaba situada en su orillas [30]. Sin embargo, tras la desindustrialización y más de 20 años de proceso de reconversión del estuario [28], la carga de contaminantes en el agua se ha reducido y la mayor parte de estos contaminantes se encuentran en las capas superiores del lecho estuarino [31], de modo que apenas apreciamos el impacto de los contaminantes sobre las comunidades de bacterias suspendidas en el agua.

La canalización o modificaciones en el paisaje tienen un fuerte impacto sobre la circulación de las aguas de los estuarios [26] y sus comunidades, donde el cambio en las corrientes natural puede provocar la aparición de nichos ecológicos nuevos. Este tipo de efectos antrópicos sobre los ecosistemas acuáticos pueden terminar siendo devastadores, como es el caso del mar de Aral que se ha convertido en una zona desértica tras el trasvase del agua de sus tributarios [32] o el caso del mar Caspio, que tras la implantación de la agricultura intensiva en las orillas de sus tributarios sus aguas se estén eutrofizando por la carga de nitratos [33]. Estos dos casos, junto con la eutrofización del mar Báltico [34,35], son ejemplo del mayor impacto antropogénico jamás ejercido sobre masas de agua marina. En el caso específico del estuario de Bilbao la canalización ha hecho que agua se eutrofice en el interior [26]. En estas aguas eutrofizadas del estuario de

Bilbao, al igual que lo hallado por Thureborn y colaboradores (2013) en las aguas eutrofizadas del mar Báltico [34], se han observado altas actividades de sulfato y nitrato-reducción, así como un incremento de la transcripción de proteínas relacionadas con la movilidad de las bacterias (flagelos) (capitulo III). Esto último se debe a que las aguas eutrofizadas suelen ser estancas y las bacterias precisan del movimiento para adquirir los nutrientes necesarios para su supervivencia. Por otro lado, las actividades sulfato y nitrato reductoras son habituales en los medios anóxicos, en donde al no existir oxígeno el sulfuro y el nitrógeno actúan como aceptores de electrones en la cadena respiratoria, generando de este modo energía.

En cuanto al impacto del EDAR, en el caso del estuario de Bilbao, está situado en las orillas del rio Galindo (capítulos II y III). En primer lugar destaca que los vertidos de los EDAR tienen un efecto notable en las aguas próximas a la desembocadura de la planta. Un fenómeno semejante fue observado por otros autores en las aguas efluentes de diversos EDAR [36-38], donde indicaban la alta presencia de bacterias propias de los tratamientos secundarios de las aguas residuales. En concordancia, en las muestras de agua recogidas en la estación de muestreo del Galindo (GAL1) se encontraron gran parte de las bacterias utilizadas en el tratamiento secundario de las aguas de los EDAR, como son por ejemplo: *Zoogloea*, *Gordonia*, *Nitrosomonas*, *Rhodoferax*, *Chloroflexus*, *Bdellovibrio* o *Nitrospira* (capitulo II) y también se han encontrado funciones activas como oxidoreductasas, decarboxilasas y comamox (capitulo III), las cuales son funciones típicas que se dan en el tratamiento secundarios de aguas de estos EDAR. Sin embargo, en el caso específico de este estuario, las comunidades y funciones descritas en la estación de muestreo del Galindo (GAL1) no se extienden por el Estuario de Bilbao (capitulo II y III). Esto se debe a que las aguas dulces del rio Galindo se quedan estancadas dentro de su cuenca, dado que su pequeña descarga no logra desplazar la masa de agua salina que entra en la boca del rio con cada ciclo mareal. Por norma general, el efecto de las comunidades de los vertidos en los ríos/estuarios está determinado principalmente por el volumen de agua que representa cada uno. En este caso, la descarga de agua del EDAR es significativamente muy pequeña comparándola con la cantidad de

agua que lleva el estuario de Bilbao a la que es vertida, por lo que su comunidad de disuelve rápidamente y se vuelve prácticamente indetectable. A esto hay que añadir que el paso del rio Galindo de agua dulce al estuario de Bilbao supone un cambio drástico a un ecosistema salino, cuyo efecto podría ser devastador para las bacterias efluentes del EDAR, e incluso para las bacterias propias del río Galindo. Por ejemplo, Drury y colaboradores (2013) observaron la pérdida de abundancia y diversidad de las comunidades bacterianas bentónicas en el rio Dupage y en el North Shore Channel de Chicago debido al efecto de los efluentes del EDAR [37]. Finalmente, tampoco podemos obviar el cambio drástico y su posible impacto en la comunidad bacteriana cuando estas se desplazan de un estanque de tratamiento de aguas a un río. Es plausible que este cambio también las obligue a cambiar su metabolismo o simplemente acaben desapareciendo a lo largo del rio por la incapacidad para su adaptación al nuevo medio. Con el objeto de evaluar el impacto de cada uno de estos cambios sobre la comunidad bacteriana sería interesante analizar las aguas del EDAR en sus diferentes instalaciones y puntos de recorrido, y de esta manera determinar cómo va evolucionando la comunidad a lo largo de todo el proceso, tal y como han estudiado otros investigadores [36,39].

Adaptación metabólica de las bacterias a su medio: el caso de *Limnohabitants*

Como todo organismo, con amplio rango de adaptación a diferentes ecosistemas, las bacterias modifican su metabolismo para adaptarse a los cambios ambientales, siempre y cuando tengan capacidad para hacer frente al cambio [40-42]. En el caso del estuario de Bilbao se ha observado que a lo largo de su gradiente salino las bacterias cambian su metabolismo con el fin de poder adaptarse a cada uno de los medios. Tal y como propusieron Fortunato y colaboradores (2015) al observar cambios metabólicos a lo largo del gradiente salino del estuario del río Columbia [6]. En dicho trabajo destacan la alta actividad de genes relacionados con la fotosíntesis

aerobica/anoxigénica provenientes del agua dulce y, por otro lado, genes relacionados con la fijación de carbono en la zona de la pluma del estuario y de la costa cercana a la desembocadura [6]. En el caso del presente estudio, tenemos que contar con un fuerte componente de anoxia y turbiedad provocada por la eutrofización en el interior del estuario de Bilbao (capitulo III). Esta situación suscita que las actividades metabólicas más activas relacionadas con la fijación del carbono se sitúen en el interior del estuario (capitulo III) en vez de en la pluma del estuario, como describían Fortunato y colaboradores (2015) [6]. Por otro lado, los genes relacionados con la fotosíntesis aparecen activos en todas las muestras, probablemente debido a la situación estacional veraniega en las que se recogieron las muestras (capitulo III). Lo que nos vuelve a demostrar que en cada estuario las comunidades se van a comportar de forma diferente, ya que la dinámica de aguas diferente va a condicionar por completo a las comunidades microbianas.

En este estudio nos hemos centrado en los cambios metabólicos del género *Limnohabitans* (*Comamonadaceae*, *Betaproteobacteria*), que es el taxón bacteriano más abundante del estuario de Bilbao (capitulo II). Este género bacteriano se ha relacionado con medios de agua dulce como ríos y lagos [43-46] y se caracteriza por englobar varios tipos de especies y cepas [45]. También, cabe destacar, que es un género bacteriano con la capacidad de crecer utilizando los exudados proporcionados por ciertas algas [44,46], potenciando de ese modo su crecimiento en presencia de ciertos grupos fitoplanctónicos. Esta puede ser la razón por la que su abundancia no desciende al pasar del agua dulce al agua salada en el interior del estuario de Bilbao, donde la concentración de clorofila es la más alta de todo el estuario (capitulo II). De esta manera, y pese a ser un género típicamente de agua dulce y sensible a los cambios de salinidad [47], su abundancia y metabolismo siguen activos en la masa euhalina del interior del estuario (B30).

Se conocen muy poco los mecanismos de adaptación a medios salinos que utiliza este género bacteriano, ya que en la práctica, totalidad de los estudios han sido realizados en ríos y lagos de agua dulce [43-51]. Por esta razón, el estudio llevado a cabo en el capítulo III sobre la alteración

metabólica que sufre este género bacteriano al cambiar de un medio con agua dulce a uno con agua salina añade nueva información relevante sobre este género. De este modo hemos descrito cambios metabólicos específicos de este género. Así, entre otros, se ha observado una activación de genes relacionados con proteínas de transporte de membrana de varios iones y péptidos, así como de genes relacionados con el movimiento celular, como son los flagelos (capitulo III). La activación de los transportadores de membrana nos puede indicar que *Limnohabitans* intenta llevar a cabo un mecanismo de regulación osmótica o de conductividad a través de la membrana como se ha observado en otras especies bacterianas [52,53]. Del mismo modo, la activación de genes relacionados con el movimiento celular puede estar relacionado con la necesidad de adquirir alimentos en una masa de agua estanca [35] o la necesidad de sobrevivir a las especies bacteriovoras [54]. Esta última hipótesis es plausible, ya que este grupo bacteriano ha sido relacionado con grandes niveles de bacteriovoria por parte de organismos protistas [45,49,55]. Enlazando con esto último, la alta concentración de microorganismos protozoos existentes en el estuario puede ser el causante primario de la disminución de la abundancia de *Limnohabitans* a medida que van descendiendo por el estuario hacia el océano, como otros autores ya han descrito el efecto de bacteriovoria por parte de protozoos en otros estuarios [54,56,57]. En visa de los resultados, no podemos descartar (1) el efecto negativo de la salinidad sobre la viabilidad de la bacteria; ya que según se observa a lo largo del estuario, a medida que el agua se vuelve más salina esta bacteria desaparece, (2) que en la medida que se aleja de la masa de agua eutrofizada, con alta concentración de elementos orgánicos (entre ellos exudados provenientes de las algas), al tener menos compuestos de los que alimentarse y al estar en un medio completamente diferente al de origen (cada vez con mayor salinidad), puede provocar un efecto devastador en su población hasta hacerla desaparecer al llegar al océano. Por estas razones, sería necesario llevar a cabo un estudio más exhaustivo, centrándonos exclusivamente en este género, para determinar la ecología de este grupo bacteriano en el estuario de Bilbao, como ya hicieron otros autores con el mismo organismo en diferentes masas de agua dulce [43-51].

Estos últimos resultados demuestran que con la implementación de forma paralela de las técnicas de metagenómica y metatranscriptómica se puede llevar a cabo el análisis funcional concreto de un grupo de organismos a lo largo de un gradiente de cambio, como pueden ser los estuarios. De esta manera podemos llegar a entender mejor las relaciones de los microorganismos con el medio, así como las modificaciones que se dan en su metabolismo a medida que el ecosistema se va transformando en otro diferente [7] para desentrañar sus mecanismos de adaptación. Así queda demostrado el potencial que tienen estas técnicas frente a las técnicas del amplicon (16S rDNA), como hemos visto a lo largo de este trabajo (capitulo II en comparación con el capítulo III), así como frente a las técnicas clásicas de microscopio y cultivo [58,59] que no permiten la detección de la mayor parte de la comunidad, y por lo tanto, de su correcto análisis e interpretación. Aun así, el alto coste de las técnicas de metagenómica y metatranscriptómica dificulta el desarrollo de estudios tan amplios en el espacio y el tiempo como pueden llevarse a cabo con las técnicas del amplicon, tal como los realizados en los capítulos I y II del presente trabajo. Por esta razón, el estudio previo del ecosistema de interés con la técnica del amplicón resulta ser interesante para focalizar el esfuerzo de las técnicas de metagenómica y metatranscriptómica de forma más eficaz.

Conclusiones generales

1.- Las comunidades bacterianas de las masas euhalinas de los estuarios de Bilbao y Urdaibai, son más estables en el tiempo que las comunidades de aguas dulces y salobres. Pese a ello, muestran una estacionalidad clara y marcada principalmente por la temperatura y la descarga del rio.

2.- Las comunidades bacterianas de los estuarios de drenaje, como es el caso de Urdaibai, pese a ser mayoritariamente oceánicas/costeras, tienen un fuerte componente de bacterias terrestres debido a la resuspensión de microorganismos que acontece, debido a las mareas, desde el fondo arenoso hasta el agua

3.- El impacto antropogénico causado por las descargas del EDAR sobre el rio Galindo está limitado al entorno de su descarga, así en en las aguas más próximas a la desembocadura del EDAR se detectan taxones y actividades metabólicas propias del tratamiento biológico de las aguas residuales, que no se extienden al estuario debido posiblemente a los cambios ambientales que sufren estas bacterias.

4.- El factor principal que determina las diferenciase entre las comunidades bacterianas de la masa euhalina del interior (B30) y las más exteriores (B33 y B35) reside en los procesos anóxicos que ocurren en el interior del estuario de Bilbao. Esto se debe a la canalización y al efecto haloclina sobre las masas euhalinas que inducen la falta de intercambio de agua después de la floración del fitoplancton, lo que provoca el episodio de eutrofización. Debido a esta situación, la comunidad bacteriana ubicada en la masa de agua euhalina del interior presenta vías metabólicas reductoras de nitrato y sulfato con el fin de reponer sus necesidades energéticas.

5.- Existen bacterias, como los miembros del género *Limnohabitans*, que son capaces de adaptarse a grandes cambios ambientales. Los miembros del género *Limnohabitans* modifican su metabolismo con el fin de sobrevivir al llegar a la masa de agua euhalina, anóxica y estanca del interior del estuario activando genes de movilidad (proteínas flagelares) y genes relacionados con el transporte de membrana y de regulación osmótica (genes de transportadores iónicos y peptídicos de membrana).

6.- El análisis en paralelo de las muestras mediante las técnicas de metagenómica y metatranscriptómica nos ayuda a comprender mejor el papel de los microorganismos en el ecosistema. Ya que no solo detectamos su presencia, si no que además podemos detectar sus modificaciones metabólicas a medida que el ecosistema se va transformando en otro diferente, y así para desentrañar sus mecanismos de adaptación. Esto contrata con los resultados obtenidos con la técnica del amplicón 16S que solo podemos detectar presencia y ausencia.

General conclusions

1.- The bacterial communities of the euhaline water masses of the estuaries of Bilbao and Urdaibai are more stable in time than the communities of fresh and brackish waters. Despite this, they show a clear seasonality and marked mainly by the temperature and the discharge of the river.

2.- The bacterial communities of the drainage estuaries, as is the case of Urdaibai, despite being mostly oceanic / coastal, have a strong terrestrial-bacteria component due to the resuspension of microorganisms due to tides, from the sandy bottom to the water column.

3.- The anthropogenic impact caused by WWTP discharges on the Galindo River is limited to the close-environment of its discharge. In other words, in the waters closest to the exit of the WWTP taxa and metabolic activities proper to the biological treatment of wastewater are detected. These taxa and metabolic activities do not extend to the estuary, possibly due to the environmental changes suffered by these bacteria.

4.- The main factor that determines the difference between the bacterial communities of the inner euhaline water mass (B30) and the outermost ones (B33 and B35) lies in the anoxic processes that occur inside the estuary of Bilbao. This is due to channeling and the effect of halocline on euhaline water masses that induce a lack of water exchange after flowering of phytoplankton, which causes the episode of eutrophication. Due to this situation, the bacterial community located in the inner euhaline water mass presents metabolic pathways reducing nitrate and sulfate in order to replenish their energy needs.

5.- There are bacteria, such as members of the genus *Limnohabitans*, that are able to adapt to large environmental changes. Members of the genus *Limnohabitans* modify their metabolism in

order to survive when they reach the euhaline, anoxic and water-tight mass of the inner of the estuary activating mobility genes (flagellar proteins) and genes related to membrane transport and osmotic regulation (genes of ionic transporters and membrane peptides).

6.- Parallel analysis of samples using metagenomic and metatranscriptomic techniques helps us to better understand the role of microorganisms in the ecosystem. Since not only detect their presence; also we can detect their metabolic changes as the ecosystem is transformed into a different, and so to unravel its mechanisms of adaptation. This contracts with the results obtained with the 16S amplicon technique that we can only detect presence and absence.

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