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Guide for the evaluation of biofouling formation in the marine environment

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Resumen

La formación del biofouling sobre estructuras sumergidas en el medio marino es una problemática mundial. Muchos de los tratamientos que se utilizan para combatir su aparición provocan efectos adversos sobre los organismos marinos y, por ello, su uso está siendo restringido. La sociedad está demandando el desarrollo de tratamientos o materiales anti-incrustantes alternativos que sean eficaces y respetuosos con el medioambiente. Así, el desarrollo de nuevos materiales en los últimos años está experimentando un auge espectacular. Sin embargo, muchos de estos materiales aún no se han validado en condiciones reales. En este contexto, el presente trabajo muestra una guía para evaluar la formación del biofouling, desde la colocación y muestreo de estructuras en dos escenarios diferentes (el pantalán de Herrera y la estación océano-meteorológica de Pasaia en el sureste del Golfo de Bizkaia), hasta el proceso completo de su análisis incluyendo el análisis del microfouling en las primeras etapas de colonización y el análisis del macrofouling en las etapas posteriores. Para ello, se muestran diferentes casos de estudio en los que se evalúa la formación del macrofouling. Así, esta guía pretende ser una herramienta útil que permita discriminar los mejores tratamientos alternativos contra la aparición del biofouling.

Palabras clave: Formación de biofouling, microfouling, macrofouling, escenarios de prueba, herramientas de evaluación, guía

Abstract

Biofouling formation on submerged structures placed in the marine environment is a worldwide problem. Many of the treatments that are used to combat biofouling provoke adverse effects on marine organisms, and thus, their use is being restricted. In this context, the society is requesting the development of alternative effective and environmental friendly antifouling treatments. The development of new materials in the last years is increasing considerably. The present work shows a guide for biofouling evaluation, from the installation and sampling of the structures in two different scenarios (the Herrera dock and the ocean-meteorological station of Pasaia in the southeastern Bay of Biscay), to the complete process of analysis including microfouling and macrofouling analysis. Different case studies are illustrated in which macrofouling formation is evaluated. This guide intends to be a useful tool that enables the discrimination of the best alternative treatments against biofouling formation.

Keywords: Biofouling formation, microfouling, macrofouling, testing scenarios, assessment tools, guide

Introduction

Biofouling or biological fouling is the non-desirable accumulation of deposits, especially living organisms, on artificial wetted surfaces. Biofouling is classified into microfouling – biofilm formation and bacterial adhesion- and macrofouling – attachment of larger organisms- (Chambers *et al.*, 2006). This latter can be 'soft macrofouling', comprising macroscopically visible algae and invertebrates such as soft corals, sponges, anemones, tunicates and hydroids, and 'hard macrofouling', formed by shelled invertebrates such as barnacles, mussels and tubeworms (Braithwaite and McEvoy, 2005).

The process of biofouling formation consists of the successive colonization of a surface by a large variety of organisms (Yebra *et al.*, 2004; Chambers *et al.*, 2006). Within the first minutes of the immersion, organic carbon residues adsorb onto the wetted surface. The primary colonization occurs within the first hours, when pioneering bacteria and diatoms settle on this modified

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surface and form the so-called EPS (extracellular polymeric substance), composed by polysaccharides, proteins, lipids and nucleic acids. The microbes together with the EPS increase the roughness of the surface, facilitating the subsequent adhesion of more particles and organisms. Hence, after approximately one week, spores of macroalgae and protozoa constitute the secondary colonizers in the process of microfouling formation. The final stage, which occurs after approximately three weeks of immersion, involves the settlement and growth of tertiary colonizers such as larger marine invertebrates and macroalgae (seaweeds) (Callow and Callow, 2011).

Biofouling formation is a highly dynamic process, varying the degree and type of biofouling depending on the geographical location, physical and chemical factors of the substrate, species diversity and environmental factors (Braithwaite and McEvoy, 2005; Greene and Grizzle, 2007; de Nys and Guenther, 2009; Jones, 2009). Among the environmental factors, temperature is undoubtedly one of the most important parameters playing a significant role in macroorganism settlement (Abdul Azis et al., 2001). This is clearly related to the fact that temperature appears to be the principal condition determining the breeding periods and rates of growth of marine animals. The amount of solar radiation is also a key factor controlling marine biofouling communities (Dobretsov et al., 2006) since it affects directly the rate of photosynthesis of the plants and thus controls the nutrition of the animals. On the other hand, pollution may also alter biofouling formation either directly through toxic effects, or indirectly, e.g., through depletion of oxygen, reduction of the solar radiation or nutrient enrichment, which may lead to a decrease in the species richness of fouling communities (Moran and Grant, 1989) or to an increase in alien species in marine hard-substrate communities (Piola and Johnston, 2009).

Biofouling causes serious problems for a wide range of marine industries and navies around the world (Yebra et al., 2004). For example, biofouling in shipping can negatively affect the hydrodynamics of a hull because the resistance to advance is higher and consequently requires a higher propulsive power and fuel consumption (Schultz et al., 2007). On the other hand, the problem concerns industries that use marine water through pipelines, as for example heating plants, gas plants and nuclear power plants (Opher and Ostfeld, 2011). The pipelines could be blocked up as a consequence of biofouling formation, diminishing the productivity and increasing the maintenance service. Biofouling not only causes problems to marine industries, but it can provoke alterations on the marine environment and on human health (Raaymakers, 2002). For example, biofouling in ship hulls is an important vector for introduction of non-indigenous species, and as such constitutes a threat to marine biodiversity (Ruiz et al., 2000; Hewitt and Campbell, 2007; Gollasch et al., 2007). Hence, biofouling derived problems imply great economic and ecological costs (Pimentel et al., 2000; Raaymakers, 2002; Schultz et al., 2011).

In order to face the marine biofouling problem, different treatments have been developed to prevent the build-up of biofouling by killing potential fouling forming organisms such as bacteria, fungi, algae, plants and mollusks (Gittens et al., 2013).

Chemical treatments or biocides are the most widely applied antifoulings, as they focus on preventing instead of on removing biofouling (Callow and Callow, 2011). However, biocides are problematic because they can leach into the environment and cause harm to living organisms other than the ones they were intended to kill (Gittens et al., 2013). For many years, the most widely used and highly effective component in antifouling paints on vessels hulls was the tributyltin (TBT) (Kiil et al., 2002). Yet, its persistence in the marine environment provokes toxicity not only on biofouling but also on other organisms, for example, inducing hormonal alterations such as imposex in certain gastropods, or shell anomalies in oysters (Ruiz et al., 1996; Rodríguez et al., 2009). Thus, the International Maritime Organisation (IMO) and Marine Environmental Protection Committee (MEPC) decided to ban the usage of TBT or other substances containing tin as biocides in antifouling paints (IMO, 2001; Regulation EC/782/2003; Dobretsov et al., 2006). In this respect, the need to develop novel approaches to environmentally-friendly antifouling treatments has become mandatory for national and international authorities (Gittens et al., 2013). Nowadays the development of new materials is undergoing a spectacular boom and as such, the topic "Innovative antifouling materials for maritime applications" is being promoted by "The Ocean of Tomorrow" through the Seventh Framework Programme of the European Commission. However, many of these new materials have not been validated under *in situ* conditions. Therefore, it is essential to have real condition installations to test the antifouling effectiveness of new materials. Additionally, standard protocols for biofouling formation measurement, from microfouling analysis in the first stages of colonization to macrofouling analysis in the subsequent stages are needed to select the most effective materials.

Hence, the aim of the present study is i) to present the scenarios available in AZTI for biofouling formation testing in fixed structures and ii) to provide a guide for the evaluation of microfouling and macrofouling formation in the marine environment. We also present different case studies of macrofouling evaluation carried out in the scenarios of AZTI. A study on microfouling formation in the same location will be published elsewhere (Rodriguez-Ezpeleta et al., in prep).

Methodology

Study areas and available scenarios

AZTI disposes of two different testing sites or scenarios within the Oiartzun estuary (southeastern Bay of Biscay) for biofouling evaluation: the Herrera dock and the Pasaia ocean-meteorological station (OMS) (Fig. 1).

The Herrera dock is located in front of AZTI building, Pasaia (UTMX, ED50, 586773; UTMY, ED50, 4797377) and represents transitional waters. Due to its high nutrient loads and high water retention time (Belzunce et al., 2004; Borja et al., 2013; Montero et al., 2013), the development of biofouling is expected to be very quick, making it ideal for screening studies. It has a depth between 2 to 7 m depending on the tide. This testing site is appropriate to
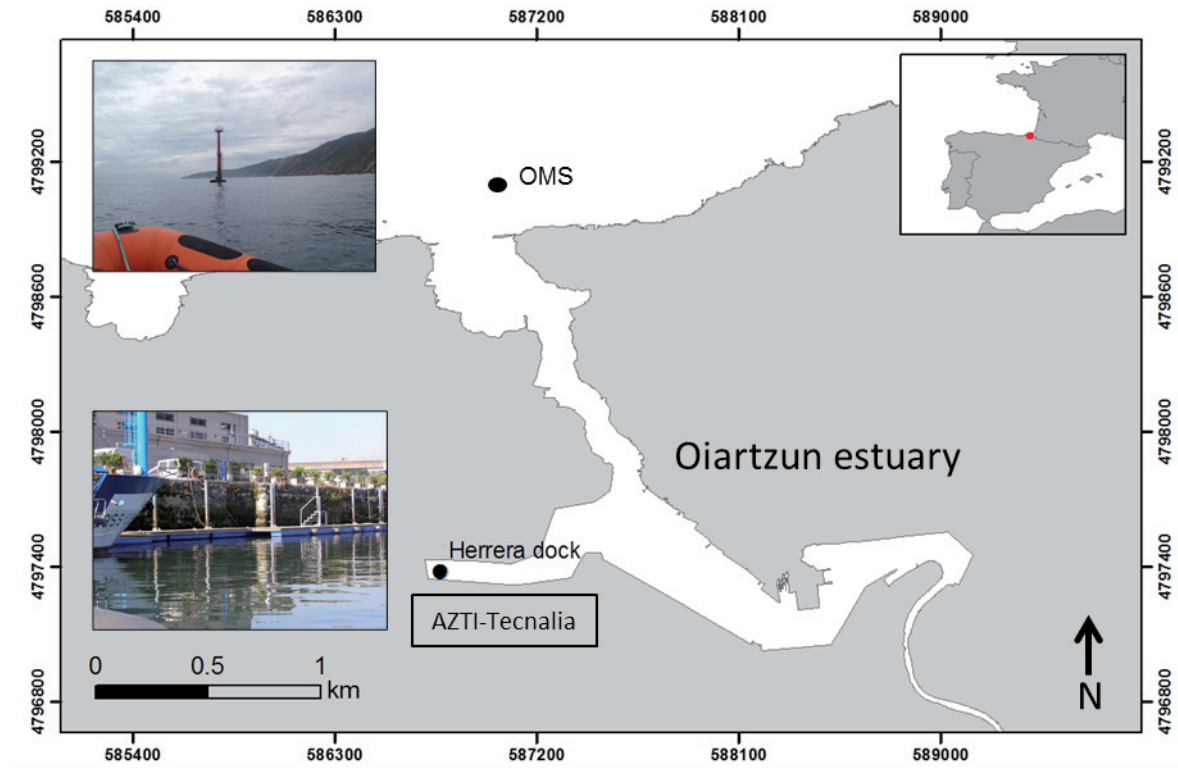
Fig. 1. Map of the study areas for the evaluation of biofouling formation showing the testing sites of AZTI at the Herrera dock and the ocean-meteorological station (OMS) of Pasaia, located in the mouth of the Oiartzun estuary.

conduct experiments in which remote sensors are required since it is supplied with electricity. Moreover, a crane situated in the dock allows placing and handling heavy structures easily. As the Herrera dock is located in a safe and easy to access zone, sampling can be carried out at any time regardless of sea conditions.

The OMS of Pasaia is located in the mouth of the Oiartzun estuary (UTMX, ED50, 586643; UTMY, ED50, 4799065) and represents coastal waters. This site is characterized by presenting high hydrodynamic energy conditions and low water residence times (Montero *et al.*, 2013), and has a depth of 20 to 25 m, allowing the evaluation of biofouling formation from the splash zone to deeper waters. Another advantage of this site is that biofouling formation can be studied in conjunction with the environmental factors determined at the OMS and in similar conditions to those found in offshore structures. However, the sampling of this testing site is subjected to sea conditions and requires expert divers.

Experimental design

Common considerations should be taken into account for an experimental design for the detection of micro and macrofouling formation. Thus, in order to obtain robust results, replicates and a control treatment should be included to validate the exposure. The Standard Test Method for Testing Antifouling Panels in Shallow Submergence (ASTM D3626-78-a, 2012) recommends the use of a slate as a nontoxic control surface since biofouling is expected

to grow easily, but a steel panel could be appropriate too. The experiment should last from days (for microfouling evaluation) to one year period (for macrofouling evaluation) depending on the type of biofouling to be determined. In the case of microfouling, the survey should be carried out daily or every two days within the first week and weekly within two months. In the case of macrofouling, as the settlement of macrofoulers requires a longer period, the survey should preferably last one year and the panels should be monitored every month. Accordingly, the dimension of the panels may vary based on the type of biofouling. For microfouling, a panel with a dimension of 10 x 10 cm should be enough while for macrofouling, a higher surface such as a panel of 25 x 25 cm is recommended. It is worth noting that these are general recommendations but each experiment could be personalized depending on specific requirements of different users.

Support structures

Based on the characteristics of each study area, the structure to support or install the panels or materials will be different. Additionally, the type of biofouling (micro or macrofouling), the number of replicates and surveys or extractions to carry out would condition the type of support structure to be used. Herein, we present different structures that may be used either in the Herrera dock (Fig. 2) or in the OMS of Pasaia (Fig. 3).

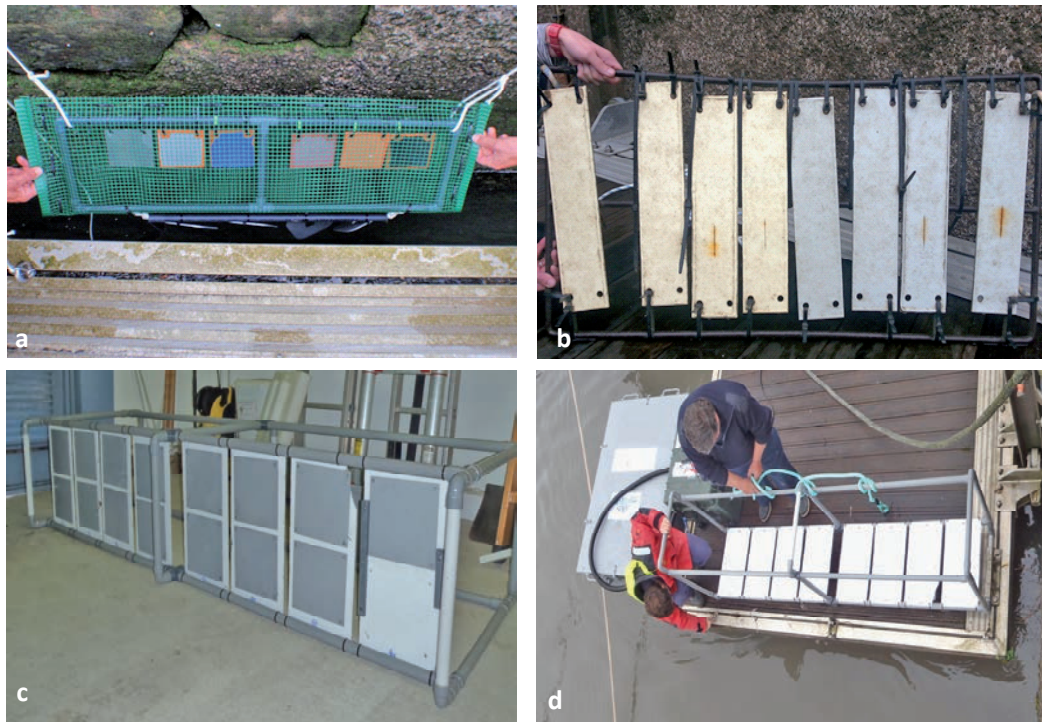At the Herrera dock, the structure can be submerged at a

Fig. 2. Sructures for the installation of different panels for biofouling formation studies at the Herrera dock. (a) A PVC structure with panels (10 x 10 cm) covered with a net to avoid predation; (b) a stainless steel structure with panels (8 x 40 cm) attached with plastic flanges; (c) preparation of a PVC structure in which panels (20 x 20 cm) are attached to plastic surfaces; (d) the same PVC structure of picture (c) placed horizontally to avoid light exposure ready to be submerged.

Fig. 3. Structures for the installation of different panels for biofouling formation studies at the OMS of Pasaia. (a) a structure consisting of two chains in which the panels are installed at the splash zone; (b) installation of the panels with shackles to the chains at the splash zone; (c) a structure composed of four chains showing panels of different size and material at a depth of 4 m; and (d) a diver attaching panels with plastic flanges to a cable at 20 m depth (OMS: Ocean-meteorological station).

variable depth of 0 to 2 m since it might be regulated by the length of a rope. The panels can be placed either vertically or horizontally to assess the light impact. The structure to install the panels can be made of different materials. However, we recommend using polyvinyl chloride (PVC) tubes or stainless steel tubes to avoid corrosion and heavy weight. It would be advisable not to exceed the weight of 20 kg to allow an easy handle of the panels. The dimension of the structure should consider the size of the panel and the number of replicates. The panels should be perforated to be attached to the structure with plastic flanges. The panels have to be placed at a certain distance without contact among them. Before placing the structure immersed under water it is important to note the order in which the panels are placed because it will facilitate the posterior identification. Additionally, the use of a net to avoid fish predation is optional.

At the OMS of Pasaia, three different structures are installed at different depths, at 25 m, 4 m and the splash zone (Fig. 3). At the splash zone and at 4 m depth, the structure to install the panels is composed by different chains arranged in parallel and attached to the cement wall. At 25 m depth the panels are supported by stainless steel cables arranged in parallel that surround the cement wall. It is worth noting that zinc anodes are placed in the stainless steel cable to avoid the attack of the electrolysis in the cable and anchor system. In order to assure a correct fixation of the panels into the structure, shackles or plastic flanges are used at the splash zone while plastic flanges are used at the submerged sites. Under water, plastic flanges are cut allowing the detachment of panels very easily. At the OMS of Pasaia the use of a net to avoid predation is not a crucial step because it is located in very high hydrodynamic waters.

Protocol for biofouling formation evaluation

a) Sampling protocol

Sampling can be performed using destructive and non-destructive approaches. To check if biofouling formation is progressing, a non-destructive approach consisting of taking photographs of a panel with a desired frequency could be performed. The picture should be taken, if possible, using always similar light and focal distance conditions and the same order to facilitate panel identification. For the qualitative analysis, the taxa that appear in the panel as well as their distribution can be grossly noted. Signs of corrosion should be also registered since these foci may alter biofouling formation. On the contrary, if biofouling formation needs to be quantified using destructive methodologies, panels should be retrieved from the water. For that, plastic flanges or shackles can be cut or removed easily. For the transport, each

panel should be kept individually inside a previously labeled plastic container full of seawater and closed tightly. It is important to avoid touching the panels to preserve all the biofouling intact. This way, in case the biofouling is detached from the panel, it will remain in the container.

Environmental parameters (salinity, temperature and dissolved oxygen) can be registered using different sensors simultaneously during the sampling.

b) Protocol for microfouling evaluation

Microfouling formation is assessed through DNA metabarcoding. This method consists on taxonomically assignment of the species present in a given sample by sequencing a standardized short DNA fragment (barcode) that is unique to each species. This qualitative methodology allows the detection and taxonomic identification of the first species colonizing a surface more rigorously than the traditional visual assessment. The process requires 1) total genomic DNA extraction, 2) barcode amplification from total genomic DNA, 3) barcode sequencing, and 4) taxonomic assignment of sequenced barcodes. Steps two, three and four are not different from any other metabarcoding study and standard protocols are well stablished for bacteria and microbial eukaryotic organisms (e.g. http://www.earthmicrobiome.org/emp-standard-protocols/). The first step, DNA extraction, is specific to each sample type and particularly critical for biofouling samples.

b.1) DNA extraction

In order to extract the DNA from the biofouling forming microorganisms, the biofilm layer should be firstly separated from the panel. This can be done by sonication or by scrapping. In both cases, detached material is suspended in a stabilizing buffer such as RNAlater® or 96% ethanol. Once suspended, the solution is centrifuged and the obtained material is used for DNA extraction (Fig. 4).

DNA extraction should be performed taking the special characteristics of biofilms into account, as some compounds such as polysaccharides present in biofouling can inhibit subsequent reactions. Extracting DNA with "standard" kits such as the *Wizard Genomic DNA Purification Kit* (Promega Corporation, Wisconsin, USA) and the *QIAamp DNA Minikit* (QIAgen, Venlo, Netherlands) will require an additional cleaning step using kits such as the *PowerClean DNA Clean-Up Kit* (MO BIO Laboratories Inc., California, USA). Otherwise, biofilm or soil specific DNA extraction kits such as the *PowerBiofilm* or *PowerSoil* kits from MO BIO are enough to obtain good quality DNA. It is very important to obtain good DNA quality (without impurities) in order to ensure
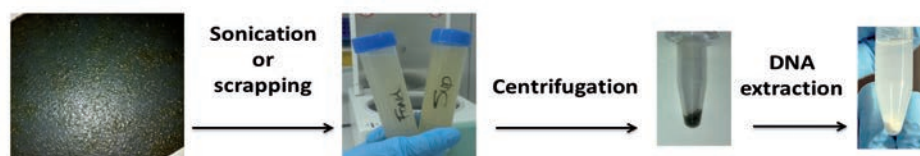

Fig. 4. Main steps for DNA extraction from a biofilm adhered to a panel.

success in subsequent enzymatic reactions (barcode amplification and sequencing).

c) Protocol for macrofouling evaluation

Macrofouling cover can be measured semi-quantitatively or quantitatively depending on the equipment available in each laboratory. However, both estimations are useful and complementary.

c.1) Macrofouling semi-quantitative cover determination

In order to semi-quantitatively assess the macrofouling cover, the percent of the colonized surface can be determined. Additionally, numerical values can be assigned following this scale: 1: for almost no patches (0-15% covered), 2: for small patches (15-50% covered) , 3: for big patches (50-100% covered) and 4: for continuous biofouling patches (100% covered).

c.2) Macrofouling quantitative cover determination

Per cent cover for each species might be estimated by superimposing a transparent grid of, for example, 250 cells on to a digital image of the panel and counting the squares under the area covered, using an image processing software such as for example Image J (http://rsbweb.nih.gov/ij/) (Fig. 5). The results are expressed as percentage of area covered on the panel (%).

c.3) Macrofouling biomass determination

Macrofouling biomass is estimated as the difference between the wet weight of the panel covered with biofouling and the initial weight of the panel (Fig. 5). In case the initial weight of the panel is not available, the wet weight of the panel after biofouling elimination can be used. The results are expressed as the weight of the area covered by macrofouling (g cm^{-2}).

c.4) Macrofouling species identification

Macrofouling species can be identified as a group of species or at species level according to World Register of Marine Species (http://www.marinespecies.org/). However, if a deeper knowledge is required, biofouling can be scraped off the panel and preserved in 4% formalin neutralized with methanol to be sent to taxonomists or preserved in 96% ethanol to be processed by molecular tools (metabarcoding). Simultaneously, the presence and the absence of

a species can be noted down, and in some cases, the census of different species can be conducted under a stereomicroscope.

Case studies

Case study 1: Macrofouling evaluation at Herrera dock

A stainless steel panel was deployed during 3 months, from 11th July to 11th October in 2012, at Herrera dock in order to determine macrofouling in terms of semi-quantitative cover and taxonomical composition, and the biological production in terms of final biomass.

The panel was removed from the water approximately every two weeks and a photogragh was taken to assess macrofouling formation. The evolution of macrofouling cover determined semi-quantitatively in the front side of the panel showed the first patches of macrofouling comprising almost the 15% of the surface after 15 days of immersion (Fig. 6). Macrofouling cover increased with exposure time reaching a maximum cover after 57 days of immersion (Fig. 6). The cover was maintained at its maximum until the end of the exposure time.
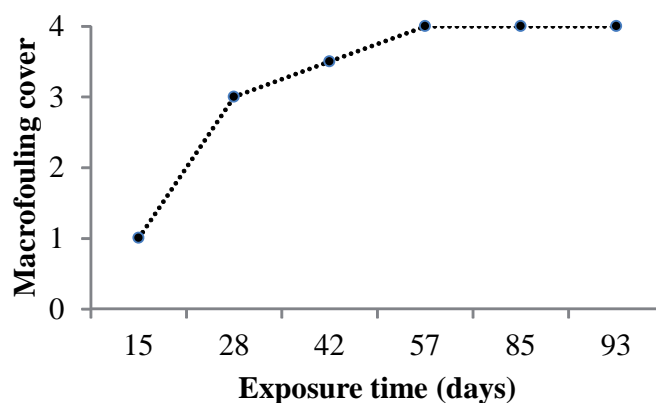

Fig. 6. Evolution of the macrofouling cover on the front side of a stainless steel panel submerged at the dock of Herrera during 3 months. Note: 1= 0-15% cover (almost no patches); 2 = 15-50% cover (small patches); 3 = 50-100% cover (big patches); and 4 = 100% cover (continuous biofouling patches).

Fig. 5. Macrofouling evaluation. (a) Macrofouling cover estimation using the Java image processing software Image J, (b) weighing a panel covered with biofouling and (c) biofouling scraping for biomass calculation.

Table 1. Identification of the main taxa that appeared in the stainless steel panel submerged at the Herrera dock from 11th July to 11th October in 2012. Note: value 0 and 1 indicate the absence or presence of one taxon, respectively.

Immersion days	0	15	28	42	57	85	93
Date	11th July	25th July	8th August	22nd August	12th Sept.	3rd October	11th October
Phallusia mammillata	0	0	0	1	1	1	1
Ascidia cf. mentula	0	0	1	1	1	1	1
Bugula sp.	0	1	1	1	1	1	1
Balanus sp.	0	1	1	1	1	1	1
Serpulidae	0	1	1	1	1	1	1

After 3 months, when the panel was removed from the water, the front side showed a macrofouling cover of 100% (4 number assigned) while the back side revealed a cover between 50-100% (3 number assigned), which indicates that the light was an important factor accelerating macrofouling growth. The macrofouling formed during 3 months showed a biomass of 3.94 g cm^{-2}. The main taxa that colonized the stainless steel panel were identified every two weeks (Table 1, Fig. 7). The cirripedes of *Balanus* genus, the bryozoan of *Bugula* genus and the chalky polychaetes of Serpulidae family appeared at first stages (after 15-28 days of immersion) while the sea-squirts *Phallusia mammillata* and *Ascidia cf. mentula* were found later (after 42 days of immersion). The presence of 5 different taxa was revealed after 42 exposure days and it was maintained along the experiment. The distribution of the macrofouling was homogenous in the whole surface but rusting signs were evidenced.
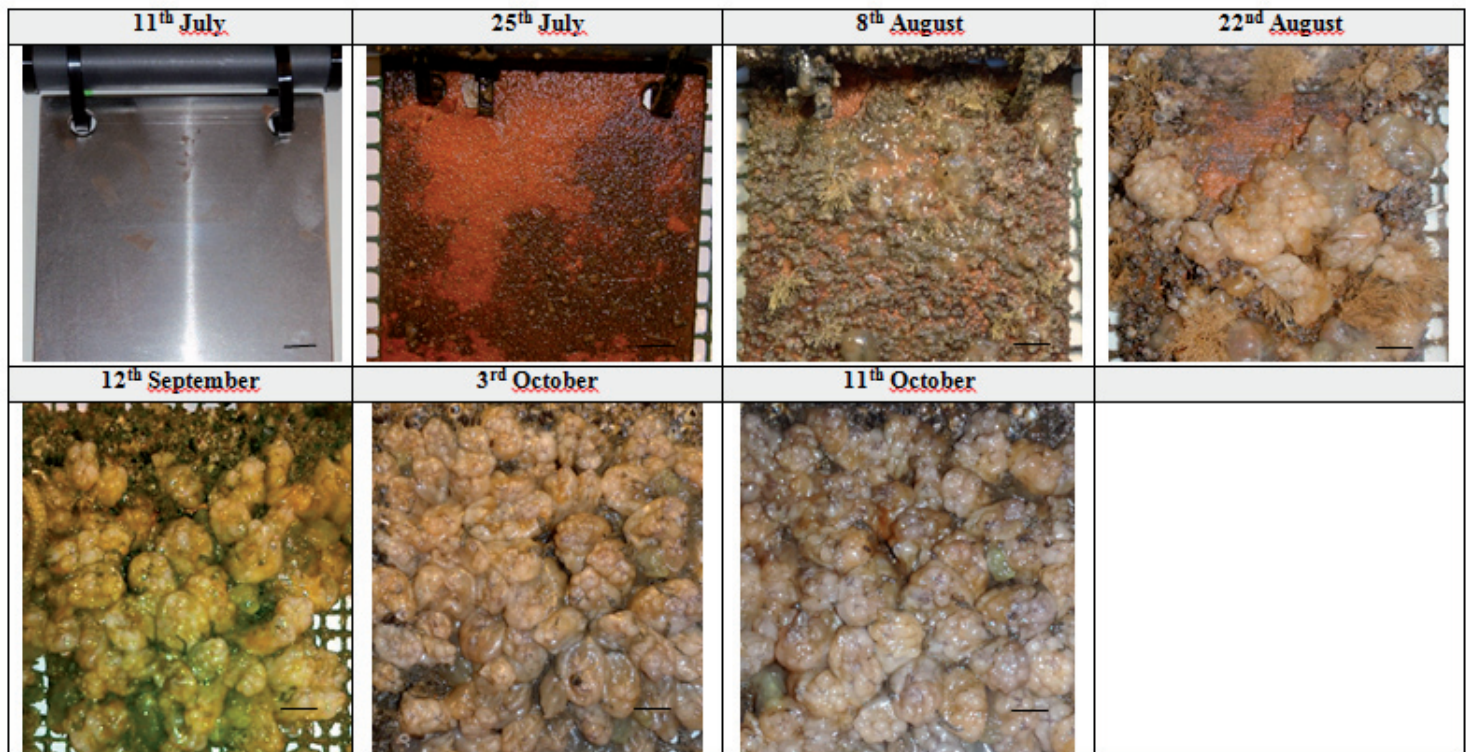

Fig. 7. Photographs showing the temporal evolution of biofouling formation on the steel panel submerged at the Herrera dock from 11th July to 11th October in 2012. Scale bar: 1 cm.

Case study 2: Macrofouling evaluation at the OMS of Pasaia

A stainless steel panel was deployed during 6 months, from 24th April to 31st October in 2013, at the OMS of Pasaia in order to determine macrofouling formation at the splash zone in terms of quantitative cover and identification of main taxa. Every approximately two weeks a photograph was taken to the stainless steel panel to analyze the macrofouling formation by non-destructive techniques. The photograph was analyzed using the Java image processing software Image J in order to assess the cover of macrofouling formation. Hence, macrofouling was confirmed after 59 days of immersion and the coverage increased with exposure time (Fig. 8). At the end of the exposure time the macrofouling showed a cover of 26% on the front side and of 11% on the back side (Fig. 9). With respect to the biomass the weight of the macrofouling was 2.86 g (0.03 g cm^{-2}) after 198 days of exposure (Fig. 10).

In relation to the macrofouling identification, a succession of different species was evidenced. Firstly the green algae appeared after 2 months of immersion, followed by the cirripedes of *Balanus* genus after 3 months (Fig. 10).
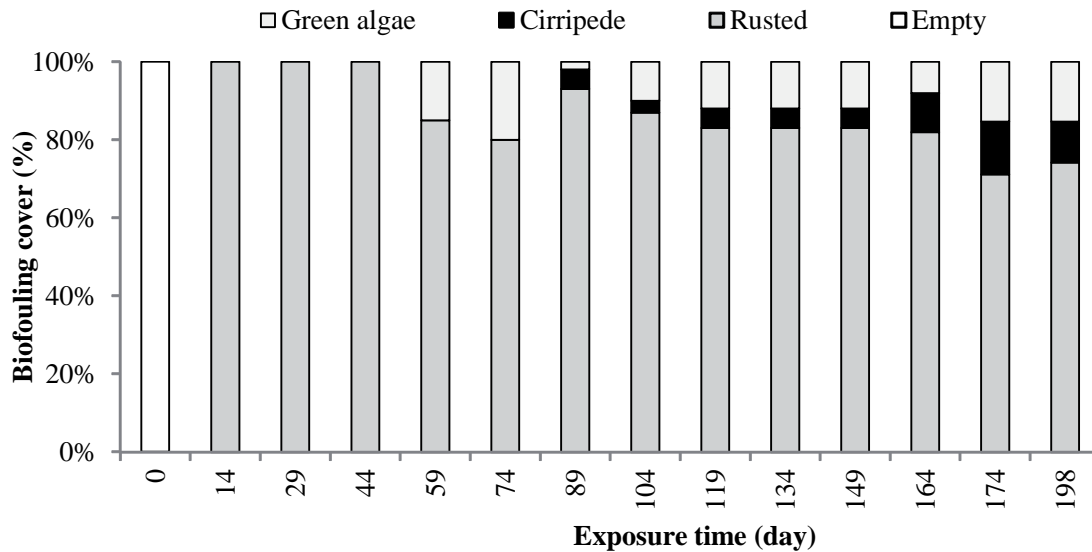

Fig. 8. Evolution of biofouling cover determined by an image processing software on the steel panel submerged in the splash zone of the OMS of Pasaia, from 24th April to 31st October in 2013.
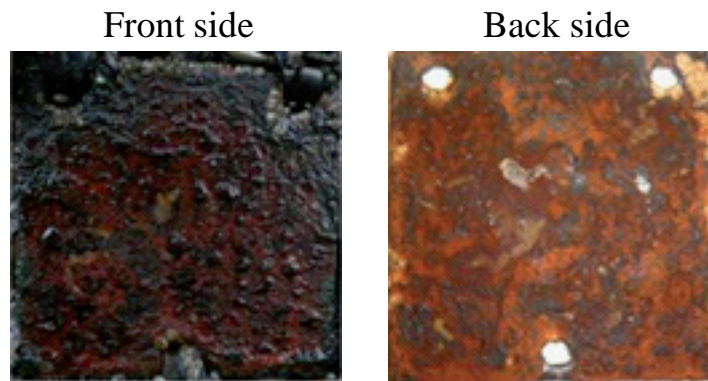

Fig. 9. Photographs showing the front side and the back side of the steel panel submerged for six months in the splash zone of the OMS of Pasaia.
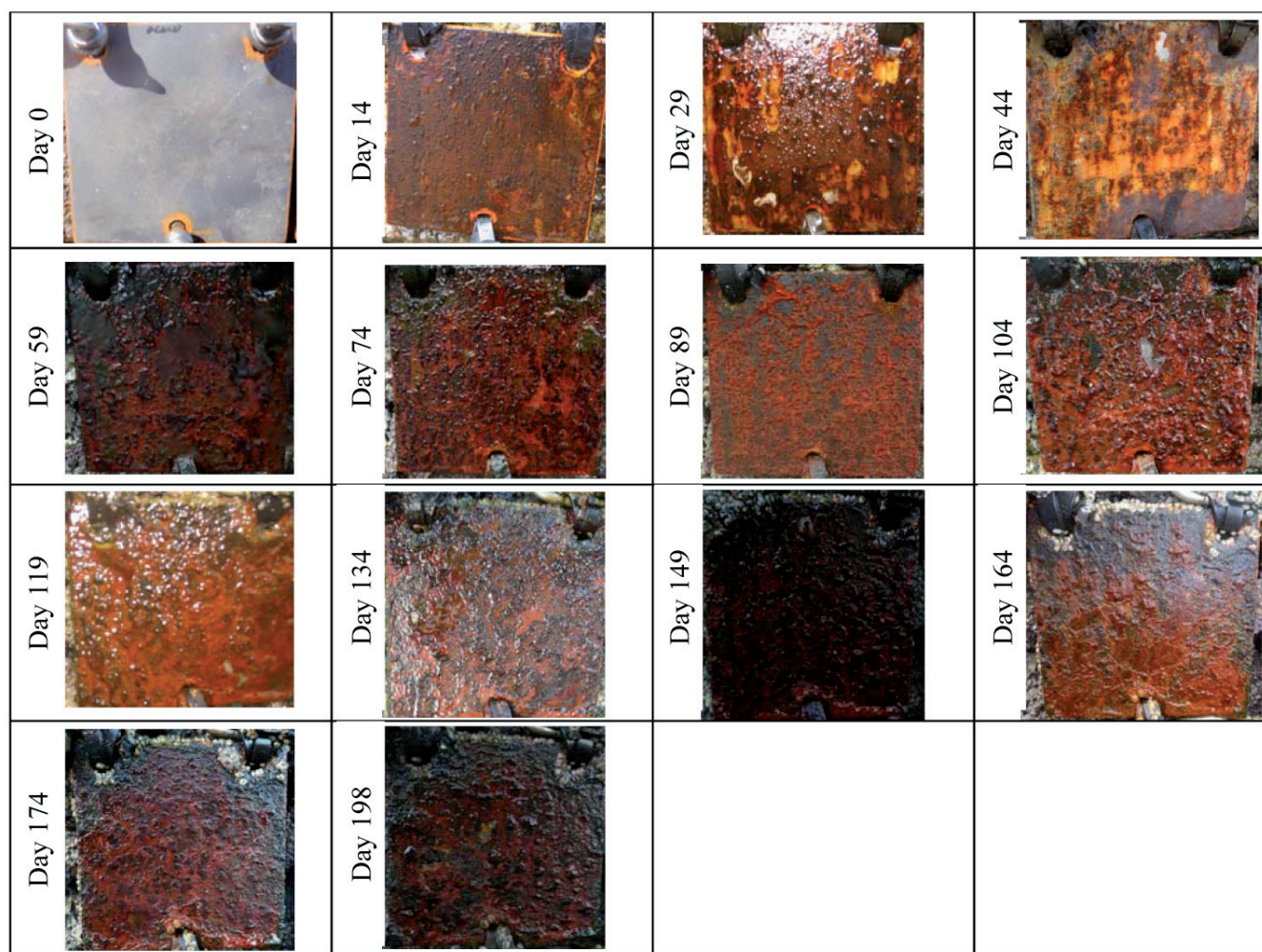
Fig. 10. Photographs showing the temporal evolution of biofouling formation on the steel panel submerged in the splash zone of the OMS of Pasaia, from 24th April to 31st October in 2013.

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