Article

Sessile Biofouling on Electrolytic Carbonated Structures: Stages of Colonization and Succession

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Abstract: The ecological succession has been widely studied by means of biofouling assemblages among different substrates, and mainly targeted in early stages on artificial ones. The present study focuses on biofouling that colonizes carbonated structures, a material similar to the natural substrate produced by the electrolysis of seawater, which is relatively very little studied. We have observed the colonization of sessile macrofouling of the port of Alicante (SE Spain, Western Mediterranean) on two types of substrates (electrolytic carbonated and steel) over 12 months of succession. The assemblages of both substrates have been analyzed by means of diversity indexes and multivariate analysis (PERMANOVA and SIMPER) in order to see the differences over time. The carbonated substrate has presented a community with higher values of biological diversity, structure and complexity, although the differences in species composition between substrates are not evident during all immersion periods. Thus, these results seem to indicate that, even after 12 months of immersion, communities are still in a dynamic successional stage.

1. Introduction

The ecological succession is a process in which a serial substitution of species takes place, with the consequent structural and functional changes in the community, after the appearance of (new) free space [1]. Regarding this, the biofouling community has been widely studied in order to understand how the primary and secondary succession processes work in the marine environment, principally in the intertidal zone [2–4]. However, during the last years, the studies are being focused in the subtidal zone [5–7]. Being aware of how the succession works is important for the correct management of marine ecosystems, not only to develop artificial reefs, but also to control the recruited organisms on the artificial submerged structures in the marine environment [5,8–10].

Many studies have been carried out about the initial stages of colonization, and it has been shown that the sequence of early colonizers will have an effect on the biodiversity and structuration of the developed fouling communities [5,11–14]. This early colonization will depend on propagule availability and environmental conditions, as most of the macrofouling organisms are driven by seasonality [1,6,15]. Another factor which influences the colonization and succession process is the substrate material. This affects directly to the species preference [16–18], generating differences in the biofouling assemblages in the early stages of colonization and maintaining them over time [6,8,19,20]. In fact, many studies have been carried out comparing biofouling communities between different substrates (natural and artificial), and they showed that the communities differ in terms

of species composition, structure, diversity and biotic interactions [5,21–23], generating even different biofouling communities within the same habitat and environmental conditions (e.g., [1,8,11,24,25]). Moreover, it has been seen that several natural materials, like sandstone, or even concrete (which can be considered as an artificial stone) may present higher biodiversity than other materials like steel [19,23,26], as they are more similar to natural rock [17,27]. In this regard, the succession process in the biofouling is affected by the time and the early colonizers, so that the events occurring during the process, principally in the early stages, will affect the final result, leading to different successional biofouling communities which will finish at different endpoints [6,28]. Nevertheless, some authors (e.g., [14,29,30]) have recorded that those different endpoints tend to converge and homogenize to the same local community. In fact, regarding the experiments performed with different substrates in the same habitat and environmental conditions, the same controversy appeared, as some authors found that communities converge [8] and others do not [19].

The substrate may cause differences within biofouling assemblages, so the type of material to choose is a relevant factor when it comes to building artificial reefs [19]. Most artificial reefs are made of artificial materials like concrete, steel, fiberglass and PVC [31], but only a few are built of natural rock, which has a higher initial recruitment than other artificial materials [16,17,27]. In fact, new concrete compositions are being developed to improve this artificial substrate's ecological properties [17,32,33]. Hence, there is a need to find materials with similar characteristics to natural rock in order to use them as material for artificial reefs or other marine structures. One of these materials is electrolytic carbonated substrate. This substrate is the consequence of the application of electrolysis to metallic materials immersed in sea water, for instance when preventing the corrosion of marine metallic structures by cathodic protection [34]. Along the process, a carbonated layer, made of $CaCO_3$ and $Mg(OH)_2$, precipitates around the cathode [35–37]. This layer is generated from the salts dissolved in the surrounding marine water, which allows this layer to be like natural carbonated rocks [31]. The potential of this material for the construction of artificial reefs was shown by Hilbertz [36], but scarce studies were performed. In fact, most of them have been carried out in tropical areas for the restoration of coral reefs [38–41] and they do not observe the colonization of other species, but corals.

Thus, the aim of this work is to observe the colonization of sessile macrofouling of the port of Alicante on two types of substrates (electrolytic carbonated and steel) over 12 months of succession. We hypothesized that the colonization and succession would be more effective in the electrolytic carbonated one as it is more like natural environment/rock. In a previous work [42], the colonization (after 3 months of immersion) of the different substrates was studied, being significantly higher in the carbonated structures. In addition, it is aimed to determine the structure of sessile community over the succession process.

2. Materials and Methods

2.1. Study Site

This study was located in Alicante's harbor (SE Spain), concretely in dock No. 9 (38°20′09.23″ N–00°29′05.81″ W). The experimental work was carried out between 15 October 2019 and 13 October 2020. The dock is located in the outer quay (Figure 1) and has a sandy–muddy seafloor with a maximum depth of 10 m. The mean surface seawater temperature varies according to the season, having a range of 14.1 to 28.3 °C, as well as salinity with a range of 37.8 to 37.3 PSU (according to https://www.puertos.es/es-es, accessed: 17 February 2024).
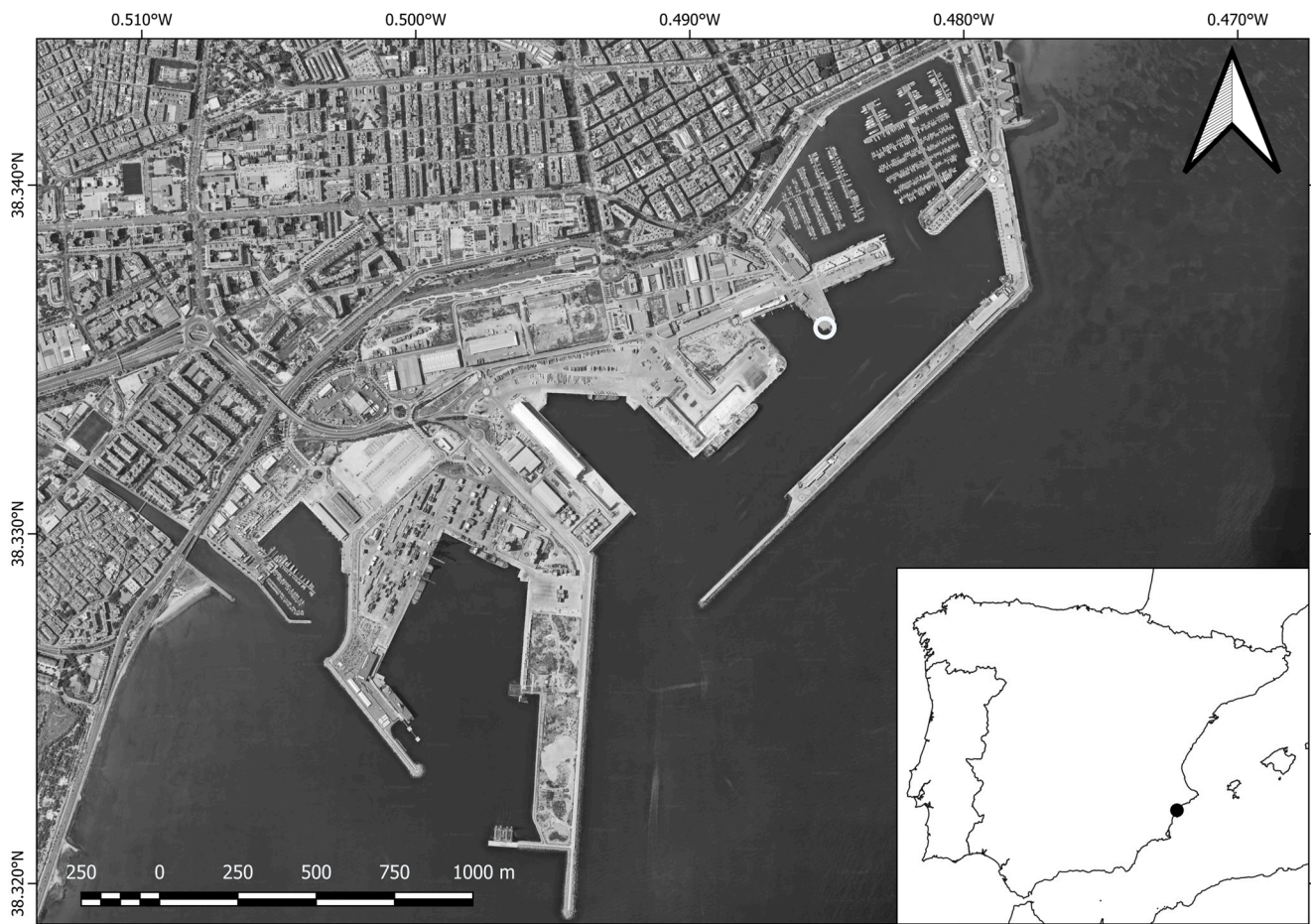
Figure 1. Alicante's harbor location and ninth dock position (white circle). Images adapted from Google Earth via QGIS 3.16.7.

2.2. Sampling

To study the colonization and succession of biofouling, 24 square steel meshes of 15 × 15 cm were used, 12 of which were previously subjected to an electrolytic process as in [36], until a 0.5 mm carbonated layer was deposited around them. In this way, 12 bare steel meshes acted as the control treatment. The meshes were arranged in three horizontal profiles of eight meshes each, four of each type, and they were placed alternately within the profile. The three profiles (all 24 meshes) were anchored at 2 m depth on 15 October 2019 with the help of three small cranes. Note that each profile was separated by 1.5 m. In order to see the colonization process, every 3 months, two meshes (carbonated and bare steel) were manually collected from each profile (a total of six meshes: three carbonated and three bare steel). These collections were made in January, March, June and October. In this way, samples were taken after 3, 6, 9 and 12 months of immersion (hereafter immersed time). The meshes were fixed with 10% formalin–seawater for at least 48 h. Then, the organisms were scraped and identified to the lowest taxon possible (based on World Register of Marine Species: https://www.marinespecies.org accessed: 15 December 2023). Only sessile macrofouling taxa were used in this work.

2.3. Data Treatment

In order to assess the effect of the substrate and the immersed time in the colonization process of sessile biofouling assemblages, both univariate and multivariate analyses were performed. In both cases, a two-way linear model was used, with substrate and immersed time as fixed and orthogonal factors, with two (carbonated vs. control) and four levels (3 vs. 6 vs. 9 vs. 12 months), respectively. It is important to highlight that the third replicate

of the carbonated treatment for the last immersed period (12 months) was lost, so the mean value of the other two meshes was used as value for that replicate.

The effect of substrate and immersed time in the diversity of the community was assessed with two-way analysis of variance (ANOVA) over the different diversity indexes calculated: species richness (S), Shannon–Wiener index (H′, 2 base), Pielou's evenness (J′) and abundance (N) organisms/cm^2 (for solitary organisms, each organism was counted, whereas for colonial organisms, each colony was treated as a single organism). In the cases that significant differences were found a Student–Newman–Keuls post hoc test was performed.

For the purpose of assessing the effect of substrate and immersed time on the colonization and succession process of biofouling assemblages, a two-way permutational multivariate ANOVA (PERMANOVA) [43] was conducted and a non-metric multidimensional scaling (nMDS) to visualize the results by using Bray–Curtis dissimilarity [44]. Note that, prior to the analysis, the data set was transformed with the fourth root, in order to reduce the weight of most abundant species. Moreover, a Similarity percentage (SIMPER) analysis was performed to detect which species contribute more to the differences and which are the characteristic ones of each substrate and period, and a permutational analysis of dispersion (PERMDISP) to test whether these differences were caused by the factor or the dispersion of the data [45]. Furthermore, a univariate PERMANOVA was performed for each species/taxon determined by SIMPER, in order to detect whether each one's abundance was significantly different between substrates. For that purpose, Euclidean distance was used when performing PERMANOVA analysis to avoid the assumption of normality of data [43]. PERMANOVA, nMDS, SIMPER and diversity index calculation were conducted in PRIMER v.6+PERMANOVA software [45], whereas the different ANOVA were conducted in R 4.1.2 [46].

3. Results

3.1. Taxonomic Study

We identified 61 different taxa during this study, most of them (53) to species level (see Supplementary Table S1). Among them, 58 (95.1%) have been recruited in the carbonated treatment and 43 (70.5%) in the control one, which means that 18 taxa were exclusive to the carbonated substrate and 3 to the control. In addition, focusing on the different submersion periods per substrate (carbonated vs. control), 29 vs. 14 taxa appeared after 3 months, 32 vs. 19 after 6 months, 43 vs. 29 after 9 months and 23 vs. 21 after 12 months. Among them, nine taxa appeared over the four periods, three are exclusive for the first immersion period, one for the second, eight for the third and six for the last one.

Looking each species in the different periods and substrates, it was found that, among the species which appear over all the immersed times, *Balanus trigonus*, *Turbicellepora magnicostata* and *Bougainvillia muscus* only meet that statement in carbonated substrate, as they are not always present in the control one. Moreover, there are species which appear in both substrates, but they need more time to be recruited in the control treatment. These species are *Crisia eburnea*, *Scrupocellaria scruposa*, *Spirobranchus triqueter*, *Hydroides dirampha*, *Parasabella langerhansi*, *Leuckartiara octona*, *Styela plicata*, *S. canopus*, *Gregariella petagnae*, *Anomia ephippium* and *Leucetta solida*.

Referring to the different taxa, Bryozoa has been the most diverse taxa (18 spp.) over the study and Hydrozoa the most abundant (mainly by three species, namely *Obelia dichotoma*, *Bougainvillia muscus* and *Eudendrium* sp.). It must be highlighted, also, that the species *Hydroides elegans* was the most abundant species (after *O. dichotoma*) during the study. Analyzing the taxa by treatments (Figure 2), there is a high abundance of Hydrozoa in both substrates during the first two periods of immersion, but in the third one (after 9 months), the abundances of the different taxa are very similar to each other in the case of carbonated treatment, while this is not happening in the control one. In fact, during that period of immersion, the highest species richness for all the taxa in both treatments were recorded. However, after 12 months of immersion, there was again a domination of

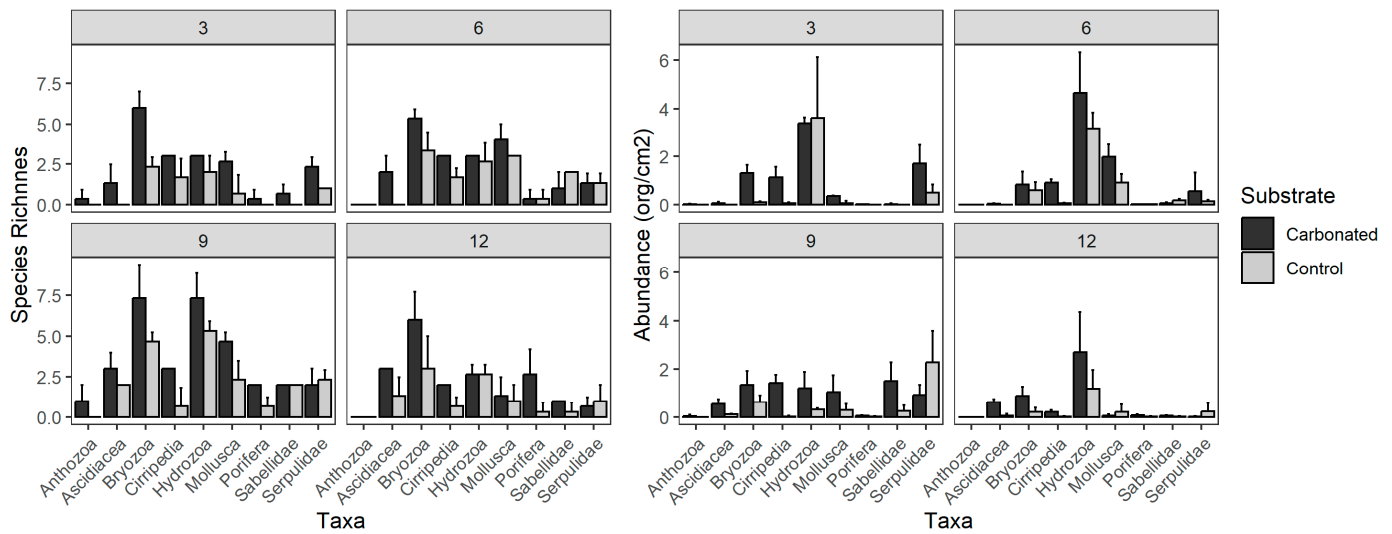Hydrozoa, in terms of abundance, and Bryozoa, in terms of richness, as in the beginning of the experiment.

Figure 2. Graphical representation of species richness and abundance (number of organisms per cm^2) for each taxon in the two substrates over the four periods of immersion: (3) October 2019; (6) January 2020; (9) March 2020; (12) June 2020. Bars: standard error. Org equals to organism.

3.2. Biofouling Assemblages

Regarding the effect of substrate and immersed time in the composition of biofouling assemblages, differences were found for both factors and their interaction (Table 1). The biofouling composition was different among substrates after 3 and 9 months of immersion, but not after 6 and 12 months, although there were marginally significant points (Table 1). These results were clearly seen within the nMDS, where different substrates were far from each other in all immersion periods, except for those immersed for 6 months, where points were very close to each other, with a high similarity (60% between them) (Figure 3). Moreover, the SIMPER analysis showed that the dissimilarities among substrates were reduced from the first to second period, but they increased in the following periods, reaching a maximum in the last period (51.91%, 38.72%, 46.2% and 62.5%, respectively). It must be highlighted that the higher dispersion was found in the control (steel) treatment (PERMDISP < 0.05), clearly seen after 12 months, when the similarity within samples was nearly 30%. However, in the carbonated treatment the similarity was always over 70%, except for the samples immersed for 6 months (65.84%).

Table 1. PERMANOVA results for the abundance of macrofouling assemblages for the different substrates and immersed times. Note: * = p-Value < 0.05, *** = p-Value < 0.001.

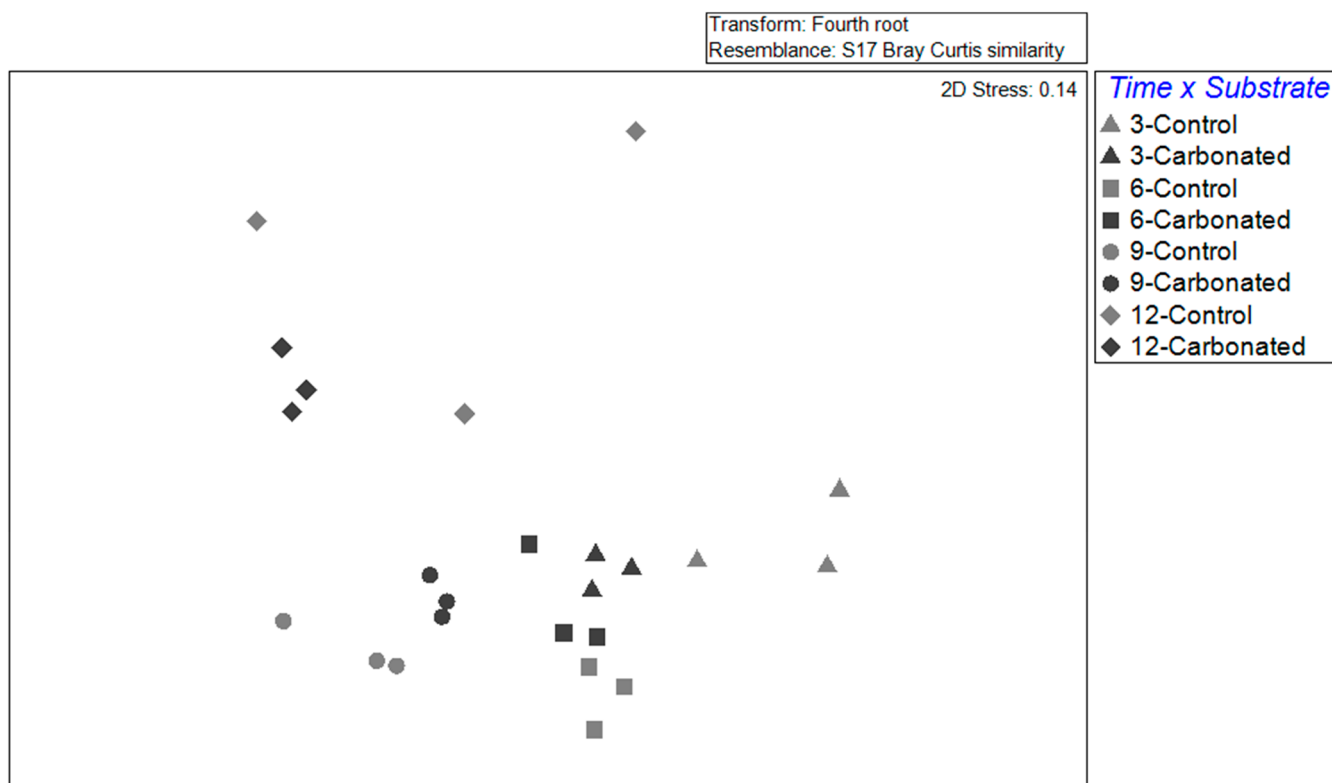| Source of Variation	DF	MS	Pseudo-F	p-Value (MC)	Post Hoc Test
Substrate = S	1	6513.1	9.2472	***	
Immersed time = T	3	7517	10.673	***	
S × T	3	1215.9	1.7263	*	3 and 9: C ≠ H
					6 and 12: C ≈ H ($p < 0.1$)
Residual	16	704.33			
Total	23				

Figure 3. A non-metric multidimensional scaling (nMDS) based on Bray–Curtis similarity of biofouling assemblages for each immersion length and substrate type.

In addition, the SIMPER analysis showed that the number of species which contributed significantly to the dissimilarity between substrates on each period varied as immersed time went on (nine, five, eleven and seven species, respectively, being always more abundant on the carbonated treatment). Thus, the characterizing species changed over the immersed times: reducing from the first to second period, increasing in the third and decreased again in the last one, highlighting a trend every 6 months. This change in species composition showed also that filter-feeders (ascidians) became typical species as the succession process continued, especially after 12 months. Among the species, *Filicrisia geniculata* and *Perforatus perforatus* stood out, as they were typical of the carbonated treatment, and they contributed to the dissimilarity among substrates in the four periods, with a mean contribution of 5.4% in each one. Moreover, another species to highlight is *Balanus trigonus*, which was also a typical species in the carbonated treatment and had the highest contribution to the dissimilarity in all the periods except for the last one. On the other hand, there were the following species, which characterized the biofouling assemblages of the carbonated treatment on each period: (i) *Schizoporella errata*, *Mytilus galloprovincialis*, *Spirobranchus triqueter*, *Turbicellepora magnicostata* and *Hydroides elegans* (3 months); (ii) *Obelia dichotoma* (6 months); (iii) *Bougainvillia muscus*, *Watersipora subtorquata* and *Sycon elegans* (9 months); and (iv) *Styela canopus* and *Botrylloides* cf. *niger* (12 months). This goes along with the results seen in Section 3.1, as the characterizing species of the biofouling assemblages were the ones which appeared over all the periods of immersion, and they had their greatest differences in terms of abundance between substrates in those periods. In addition, for the cases of *S. elegans* and *B.* cf. *niger*, they only appeared in those periods.

3.3. Biofouling Diversity

Referring to biodiversity, all indexes and abundance were statistically higher in the carbonated substrate (Table 2 and Figure 4). In addition, a temporal trend can be seen, where the species richness and Shannon–Wiener index were higher after 9 months of

immersion than in any other period, whereas the abundance was kept similar during the first three periods, before decreasing in the last. Pielou's evenness was not affected by the immersion length.

Table 2. ANOVA results for each diversity index and abundance of biofouling community for both substrate and immersed times. Note: * = *p*-Value < 0.05, ** = *p*-Value < 0.01, *** = *p*-Value < 0.001.

|	**Source of Variation**	**DF**	**MS**	**F**	***p*-Value**	**SNK**
S	Substrate = S	1	570.4	62.507	***	C > I
	Immersed time = T	3	192.4	21.082	***	9 > 3 = 6 = 12
	S × T	3	14.5	1.588	0.231	
	Residuals	16	9.1			
H	Substrate	1	5.894	24.935	***	C > I
	Immersed time	3	1.912	8.088	**	9 > 3 = 6 = 12
	S × T	3	0.424	1.794	0.188788	
	Residuals	16	0.236			
J	Substrate	1	0.07071	6.512	*	C > I
	Immersed time	3	0.02659	2.449	0.1012	
	S × T	3	0.01403	1.292	0.3113	
	Residuals	16	0.01086			
N	Substrate	1	78.94	36.596	***	C > I
	Immersed time	3	15.78	7.314	**	12 < 3 = 6 = 9
	S × T	3	0.7	0.326	0.80647	
	Residuals	16	2.16			
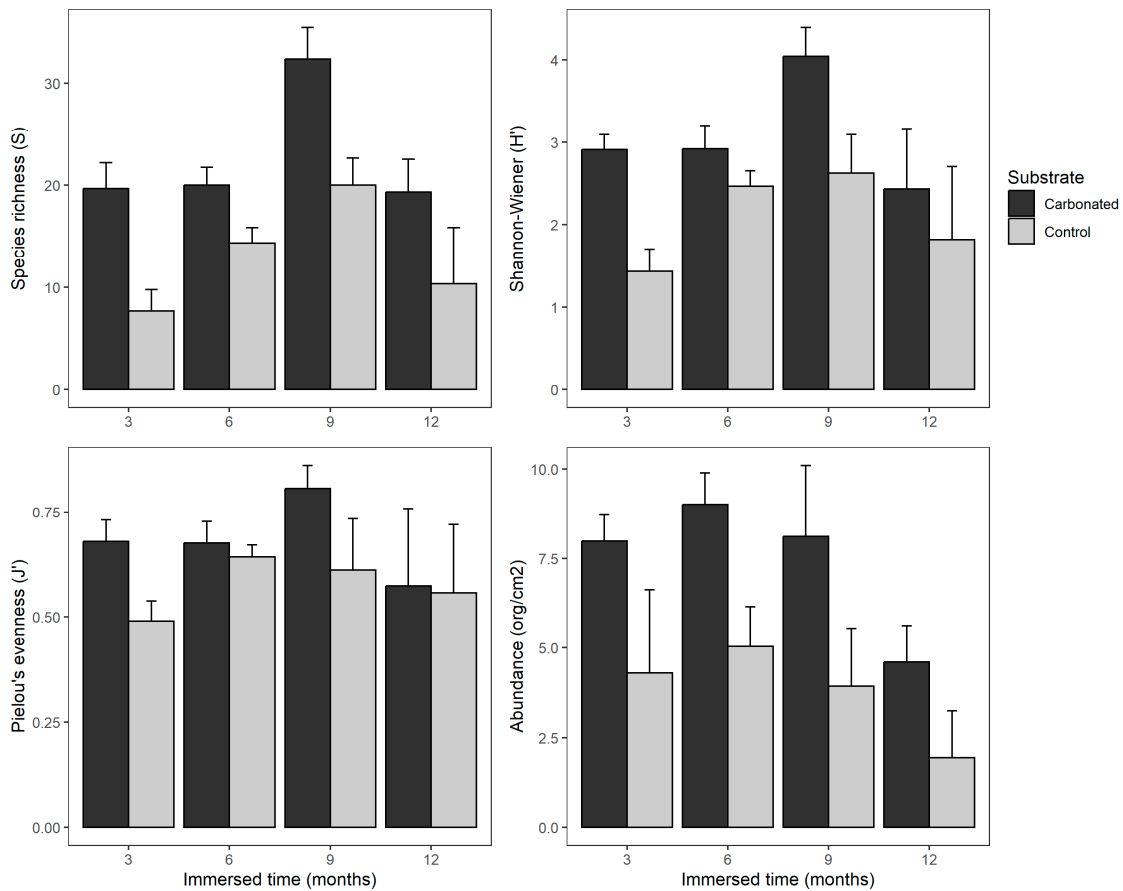
Figure 4. Bar graph for the different diversity indexes measured and abundance (number of organisms per cm^2) for each substrate and immersion length (months). Bars: standard error.

4. Discussion

The substrate caused differences in the biofouling assemblages during the early colonization stage and during the succession process. This is supported by several studies in which the assemblages differed between substrates as succession develops [17,19]. However, in this work, there are periods in which the differences are not clear, suggesting that the biofouling community still remains in a dynamic state, and it did not reach final succession stage, as it did in [6], after being immersed for one year in the Central Mediterranean. In fact, a clear seasonal pattern was observed, in which, every 6 months, the biofouling assemblages tended to converge and then diverge again (only tested for first 6 months, as the study finished after 12 months). These periods corresponded to winter and summer, when recruitment rates were lower and higher, respectively [1,6,14,15]. Nevertheless, both periods have been characterized by a dominance of hydrozoans (*Obelia dichotoma* and *Bougainvillia muscus* after 6 months and *Eudendrium* sp. after 12). Hydrozoans are erect, stolonal and fast-growing species, which gives them the ability to colonize the empty space easily and grow over other species. In fact, this suggests they can exploit the least suitable seasons to colonize better than other species, and, during winter, they can use the space left behind by algae as there is less available light [47]. Furthermore, once the unfavorable winter months left, the diversity of both substrates increased and the differences in biofouling composition appeared again. One reason to explain this is the arborescent three-dimensional growth of hydrozoans, which increases the complexity and colonization of both vagile and sessile species for the next periods. Indeed, it has been proven that they can easily recruit mytilids and some solitary ascidians; among others, this occurred in [47–51] as it happened in this work: the appearance of *Mytilus galloprovincialis* after 6 months and Styelidae juveniles after 9.

There is no doubt that the presence of propagules in the environment determines the appearance and abundance of certain species relative to others [14,52], and it was observed that species were substituted during the succession process. In fact, this propagule availability in the environment has a seasonality which allows or facilitates the recruitment of some species over others [14,53], leading to different biofouling communities depending on the time of immersion, as reported in Lezzi and Giangrande [6]. However, some species were characteristic of carbonated treatment, appearing in all or almost every period on it (*B. trigonus*, *F. geniculata* y *P. perforatus)* which means that their propagules were abundant in the study area, and they can act as pioneer species and persist on that substrate over time. In fact, these species have a calcareous skeleton, and [54,55] and [26] showed that cathodically protected structures and calcareous substrates have a higher recruitment of barnacles, agreeing with this finding.

On the other hand, a substitution of species was observed as the succession went ahead, where active filter-feeders (mainly ascidians) became more important, particularly at the end of the experiment. The dominant ascidians were the typical ones of port environments and during the summer months [51,56]. In the case of ascidians, it has been reported that their larvae prefer rough surfaces to settle [21,57] explaining why they are more abundant on the carbonated substrate. Moreover, during this species substitution process, there are species, such as *Crisia denticulata*, *Savignyella lafontii*, *Botrylloides* cf. *niger*, *Ostrea stentina*, *S. ciliatum* and *Sycon* sp., which appeared only after 12 months. This finding is explained by the seasonality of the species (as in the case of *B.* cf. *niger*, which is a typical species of warm periods) or by the need of a developed pioneer community to settle, as in the case of *Sycon* spp., which need a developed algal community [58]. In addition, a lag in the recruitment of certain species (*Scrupocellaria scruposa*, *Spirobranchus triqueter*, *Hydroides dirampha*, *Parasabella langerhansi*, *Leuckartiara octona*, *Styela plicata* and *S. canopus*) can be observed between both substrates. These species took at least one trimester longer to be recruited in the control treatment, which reinforces the fact that the carbonated substrate has higher recruitment and diversity rates. This may be due to the fact that the pioneer community developed more rapidly in the carbonated substrate, not only because of its greater capacity for initial recruitment [42], but also because of the greater abundances of

hard-shelled organisms, such as balanids and mytilids, and hard-bodied organisms like solitary ascidians, among others. These organisms are able to generate more space to be colonized by other organisms, thus increasing the capacity for recruitment of new species over time. Furthermore, as recruitment is also dependent on the presence of propagules in the environment, the gap in the recruitment among both substrates could be explained by the enhancement of propagules from the species that developed in the carbonated treatment, which were easier recruited in the carbonated meshes.

The higher biological diversity values were obtained in the carbonated treatment, being significantly higher after 3, 6 and 9 months, and decreasing in the last period. These findings are consistent with other studies [19,23,26] that found higher biological diversity values in natural rock and concrete substrates that were similar to electrolytic carbonated substrates. Moreover, the results are comparable to studies conducted by Neves et al. [59] and Albano and Obenat [23], in which calcareous substrates, such as concrete, had a higher abundance of organisms than other substrate types because their surfaces are similar to natural rocks. The differences found among substrates are consistent over time from the beginning of colonization, indicating that the carbonated substrate is capable of generating a more structured and developed fouling community in less time than steel. Nevertheless, a drop in the diversity and abundance of organisms has been observed after 12 months of immersion, matching with a certain homogenization of the assemblages, possibly due to greater competition among species due to substrate limitation [5,60]. It was in this period when the higher dissimilarities between substrates were found and a dominance of *Eudendrium* sp. was detected in both substrates. The remaining dominant species differed according to the substrate, with arborescent bryozoans and ascidians (mostly solitary) predominant in the carbonated one and hydrozoans and serpulids (*H. elegans*) in the control. The dominance of these fast-growing and high space colonizer fouling species [61] may explain the decreasing of indexes and the convergency, rather than statistical differences, of assemblages in this period. However, it was expected that the diversity and the abundance of organisms will continue increasing, due to a major propagule supply enhanced by the warming of seawater [1,6,14,62,63]. Nevertheless, the effect of higher temperatures during the last period of sampling (the meshes submerged for 12 months) could also explain the decreasing of abundance of fouling species as some species cannot cope with them, mainly the ones dominant during the cold months.

As has been explained, the dissimilarities within the biofouling assemblages in the substrates were high and there was not a clear homogenization of communities among both substrates after 12 months of immersion, as there was when the dissimilarities were higher. Hence, it indicates that the communities did not reach an endpoint. So, a longer-term study is required, as conducted in other works (e.g., [8,19]) to elucidate where the communities reach and how the succession process develops on both substrates. In fact, it could be hypothesized that, as carbonated treatment has more dead and alive hard-bodied species, once the warm period has ceased, the largest space availability of that substrate will be easily and rapidly colonized by species. In addition, considering the seasons, a complementary work is suggested by the authors in order to see the effect of immersion time (season) on both early colonization and succession over the two substrates, as was carried out in [6,14,15], and to determine the seasonal peaks of the species from Alicante's harbor.

Based on the above, the carbonated substrate presented a community with higher values of biological diversity, structure and complexity, although the differences in species composition between substrates were not evident during all immersion periods, which seems to indicate that, even after 12 months, the communities were still in a dynamic successional stage.

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