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ORIGINAL RESEARCH ARTICLE

An eco-friendly strategy using flax/polylactide composite to tackle the marine invasive sponge *Celtodoryx ciocalyptoides* (Burton, 1935)

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Summary Discovered in the 1990s in the river of Etel (Morbihan, France), the marine invasive sponge *Celtodoryx ciocalyptoides* originating from the Chinese Yellow Sea is now well implanted on concrete pilings inside the Etel marina (Morbihan, France). Novel eco-friendly strategies are urgently needed in order to limit its adhesion on concrete and the risk of dispersal outside the marina. In this study, the anti-settlement and anti-attachment properties of flax/PLA, a biocomposite made of polylactide reinforced with flax fibres, were evaluated on sponge propagules' behaviour. First, flax/PLA panels were immersed into the Etel marina for six years. The coverage onto PLA panels of marine invertebrates was estimated twice a year. In a second step, PLA panels were used as artificial support for invasive sponge transplants. In comparison, specimens were transplanted in mesh bags. Sponge weight increase was measured twice a year. Results indicated that the occurrence of the invasive sponge was delayed for two years on biocomposite in comparison with concrete. At the end of the six-year study, macrofouling by marine invertebrates did not exceed 70% of the surface of the panels and no *C. ciocalyptoides* specimens were observed. Once transplanted on PLA panels, sponge specimens were able to survive the first year without growing. After two years, none of the transplants survived while specimens in mesh bags increased their weight by 100%. These findings highlight the potential interest of biocomposite in the development of coastal and marine infrastructures.

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1. Introduction

Discovered in 1996 in the Etel River (Brittany, France), the marine sponge *Celtodoryx ciocalyptoides* originating from the Chinese Yellow Sea is now well established and considered as an invasive species (Henkel and Janussen, 2011; Perez et al., 2006). In the Etel marina, this species was recently shown to cover on its own up to 17.4% of the surface of the concrete pilings (Gentric and Sauleau, 2016). The species is also present in the Gulf of Morbihan (Perez et al., 2006), in the river of Penerf (Sauleau, Personal communication), in the Oostershelde (Netherlands) (Van Soest et al., 2007) and has recently been observed in the harbour of Le Havre (Normandy, France) (Berno et al., 2016).

One possibility to limit invasive species adhesion on artificial hard substrate is the use of antifouling paints. Copper-based antifouling paints are applied on immersed structures to limit the growth of fouling organisms. However, by reducing biodiversity, competition with endemic species, and by inducing tolerance to copper pollution, antifouling paints are thought to enhance invasions (McKenzie et al., 2012; Piola et al., 2009). Thus novel antifouling strategies such as the use of self-polishing matrices for controlled release of natural antifouling substances were developed (Thouvenin et al., 2003).

Among self-polishing polymers, polylactide (PLA) is probably one of the most promising erodible polymers due to the fact that PLA is biodegradable, recyclable, compostable, biocompatible and eco-friendly produced. PLA is obtained using the ring-opening polymerization of lactide, a cyclic monomer derived from plant resources (corn starch, cane molasses, etc.). PLA showed relative biocompatibility as suture material, orthopaedic devices and drug release delivery systems for medical application (Farah et al., 2016; Hamad et al., 2015; Ulery et al., 2011; Walczak et al., 2015). In contrast to traditional highly durable plastics, PLA showed good biodegradability in soil and compost (Hakkarainen et al., 2000; Karjomaa et al., 1998; Pranamuda et al., 1997; Sukkhum et al., 2009). The mechanism of degradation includes both chemical hydrolysis and biodegradation. The polymer initially degraded through abiotic hydrolysis leading to the release of shorter oligomer chains and monomers of lactic acid (LA). This chemical hydrolysis is followed and accelerated by biodegradation (assimilation) by micro-organisms leading to carbon dioxide and water.

The direct contribution of the biodegradable polymer to the overall antifouling effect was recently investigated by Ishimaru et al. (2012) who prevented the attachment of barnacle cypris larvae using a polyethylene/PLA blend. Among hydrolysis products, LA was suggested to participate in the anti-barnacle attachment property of PLA. In addition, LA is known to have antibacterial effects by reducing the pH while low molecular weight PLA were suggested to have prolonged antimicrobial effects (Ariyapitipun et al., 1999). The use of bacteriostatic PLA against *Pseudomonas aeruginosa* and *Staphylococcus aureus* was also proposed in the conception of tympanostomy ear tube for children (Ludwick et al., 2006). Eventually, PLA microspheres were shown to be slightly bacteriostatic towards a *Pseudoalteromonas* sp. (Faÿ et al., 2008) while PLA film may have bacteriostatic activity against *Salmonella typhymurium* (Theinsathid et al., 2012).

It is well known that biofilms composed of bacteria, diatoms, protozoa or fungi are the prerequisite for the settlement of invertebrate larvae (Qian et al., 2007; Whalan and Webster, 2014). Thus, the hypothesis of our study was that the biodegradable and/or potential antimicrobial properties of PLA could be used as an eco-friendly biomaterial to limit propagules settlement. In order to assess the anti-settlement properties of PLA, biocomposite panels made of polylactide reinforced with flax fibres were immersed into the Etel marina for six years. The coverage onto those panels by the invasive sponge and other marine invertebrates was estimated twice a year. Since sponge overgrowth occurs also by lateral colonization, we evaluated the anti-attachment effects of flax/PLA on *C. ciocalyptoides* transplants bound to the biocomposite panels for the same period of time. As far as we know, this is the first time an eco-friendly strategy is developed to get rid of an invasive sponge on the North-East Atlantic coast.

2. Material and methods

2.1. Study site

Experiments were performed from April 2011 to March 2017 in the Etel marina (47.659°, −3.207°) located at the mouth of the river of Etel (Brittany, France). This river, 15 km long, is located between Lorient and Vannes (Morbihan, France) and belongs to the Natura 2000 network areas (FR5300028).

2.2. Sponge collection

Sponge samples were collected by SCUBA diving at a depth of 10—15 m during neap tides in April 2011 along the Magouër site situated 200 m west of the marina. *C. ciocalyptoides* specimens freshly collected were immediately transferred to the marina for transplantation. Samples were cut in approximately 125 cm^3 pieces, weighed, tagged, and let in a nylon mesh bag for control or separately bound onto a flax/PLA panel with an iron wire (Fig. 1).

2.3. Experimental set-up and design

Poly(L-lactic acid) (PLLA, Biomer® L9000) was purchased from Biomer (Germany). The polymer has a high molecular weight (220,000 g mol^{-1}) with a L- and D-isomer ratio of approximately 98:2. Flax fibres of the Marylin variety (1 mm length) were incorporated in PLA polymer (20% in weight) as previously described (Le Duigou et al., 2014).

The support structure (1 m^3) was made with PVC tubes (25 mm diameter). Each face was divided in five columns with a nylon rope, strengthened every 20 cm, each length containing 5 replicates i.e. 5 nylon mesh bags (36 × 29 cm, 3 mm mesh). Each mesh bag contained two sponge specimens, one let free into the net and another one bound with an iron wire to the panel (9.8 cm × 14.7 cm × 0.18 cm) made of flax/PLA. Both specimens were separated to avoid fusion. A total of 80 mesh bag were fixed to the 4 PVC quadrats linked each other. On the top of the structure, additional panels made of flax/PLA were attached all around the piling for settlement experiments. Panels were immersed at a depth of 9 m below sea level and fixed around the north-western piling of the

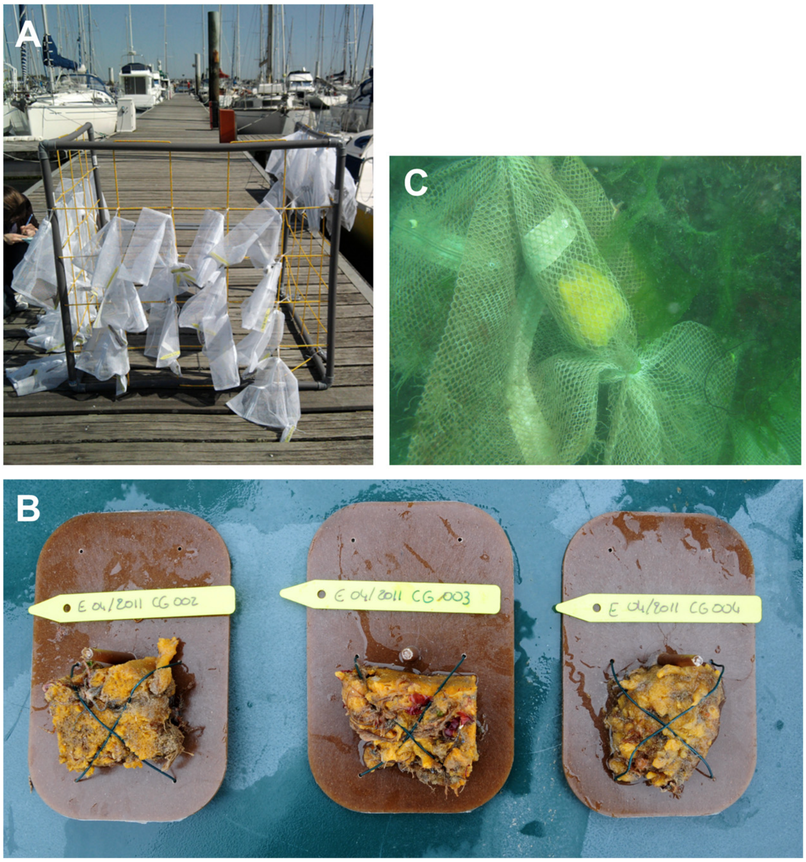

Figure 1 (A) The support structure. (B) *Celtodoryx ciocalyptoides* samples (125 cm^3) bound with an iron wire to tagged flax/PLA panels. (C) Mesh bags containing two sponge specimens: one let free inside the net and the other one bound separately onto the PLA panel.

marina. To respect hydrodynamic conditions, the distance between the piling and the support structure was 30 cm.

In order to assess sponge capacity to colonize any artificial substrate which may vary according to life cycle, the fauna covering a surface of 0.1 m^2 at 7 m deep was scraped off the concrete piling (Fig. 2a) at the beginning of the study. Sponge settlement on this area was observed in situ every 6 months by underwater pictures (Canon G10).

2.4. Sponge monitoring

In order to assess sponge survival and growth after transplantation in a new marine environment, percentage weight increase and elemental composition were measured. Five mesh bags were sampled by SCUBA every 6 months. Fresh sponge samples were gently removed from the net, directly weighed on the pontoons of the marina, bring back to the lab and kept at -80°C before lyophilization.

The percentage weight increase was calculated with the formula:

$$\%\,\text{Weight Increase} = \frac{(W_m - W_0)}{W_0} \times 100,$$

where W_m is the sponge weight after m months of growth and W_0 the sponge weight at the beginning of the experiment.

The following Metallic Trace Elements (MTEs) Ba, Cd, Co, Cr, Cu, Fe, Mg, Mn, Mo, Ni, Si, Sr and Zn which are vital for the growth and development of sponges were quantified in transplanted specimens and compared to wild specimens collected outside the marina from the natural site (Magouër). The elemental content was determined every year during a monitoring period of three years (2012, 2013 and 2014) by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) by the University of Caen as previously described (Mahaut et al., 2013). Analyses were performed in triplicate on a mixture of 5 sponge specimens.

2.5. Covering and growth on PLA panels

The presence of micro- and macro-foulers on both faces of the flax/PLA settling panels ($n = 10$) was observed every 6 months by Scanning Electron Microscopy (SEM) (JSM-6460LV, Jeol) and underwater pictures, respectively. Spicules characteristic of the skeleton of *C. ciocalyptoides* were observed by SEM. Percentage cover of macrofoulers was calculated by using ImageJ software. Sponge growth was estimated by calculating the percentage weight increase as described in Section 2.4.

2.6. Statistical analyses

Transplants weight increase under the two conditions (on flax/PLA plates or on nylon mesh bags), MTEs composition between wild and transplanted specimens, and seasonal

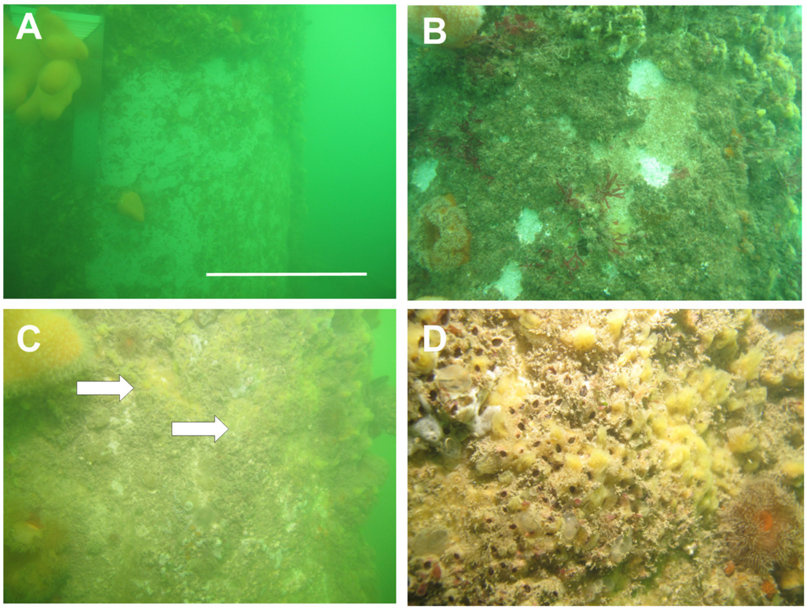

Figure 2 Fouling by the marine sponge *Celtodoryx ciocalyptoides* on concrete piling within the Etel marina. (A) A 0.1 m^2 area was scraped on the piling (scale bare = 25 cm). (B) The scraped area was rapidly covered by mainly barnacles after 6 months and (C) fouled by *C. ciocalyptoides* specimens (white arrows) after 1 year. (D) A zoom showed the sponge cover on the shells of barnacles competing with the hexacorallian *Metridium senile* and the tunicate *Diplosoma spongiforme*.

covering rate between spring and winter were compared using Wilcoxon-type test. All analyses were performed with Rstudio 1.0.143.

3. Results

3.1. Anti-settlement properties of the flax/PLA biocomposite

During the first 6 months of this study, the fouling at the surfaces of the panels was dominated by microfoulers such as diatoms, bacteria and hydrozoans (Fig. 3). In some cases, few macrofoulers such as tube worms *Pomatoceros* sp. and barnacles *Balanus* sp. were observed on both faces of the PLA panels (Fig. 4a and b) covering up to 4.6 ± 0.3% of the area (Fig. 5). In comparison, the PVC structure was covered of hydrozoans and tunicates such as *Ciona intestinalis* (Fig. 4a). During the next two years of immersion, both surfaces were partially (37.5 ± 8.7%) covered of mainly bivalves *Anomia* sp., mussels, tube worms *Pomatoceros* sp., few anemones *Metridium senile*, and tunicates but no *C. ciocalyptoides* specimens were observed (Figs. 4c and 5). After three years (Fig. 4d), half of the surface of PLA panels (48.7 ± 7.2%) were covered mainly of *Balanus* sp., *Anomia* sp. and *Mytilus* sp. In few cases, *Crassostrea gigas* and *Crepidula fornicata* were also observed. Sponge cells with spicules consisting part of the skeleton of *C. ciocalyptoides* (Fig. 6) were finally observed onto barnacle shells covering up to 25.5% of the panel. After 6 years of immersion, the fouling covered more than two thirds of the panel surface (70.6 ± 7.6%) but neither invasive sponge specimens nor barnacles were observed (Fig. 4e). During the six-year study, the percentage cover of macrofoulers did not decrease significantly each winter (p-value > 0.05) (Fig. 5).

3.2. Anti-attachment activity of the flax/PLA biocomposite

After one year, the weight of sponge transplants bound with an iron wire onto the flax/PLA panels severely dropped

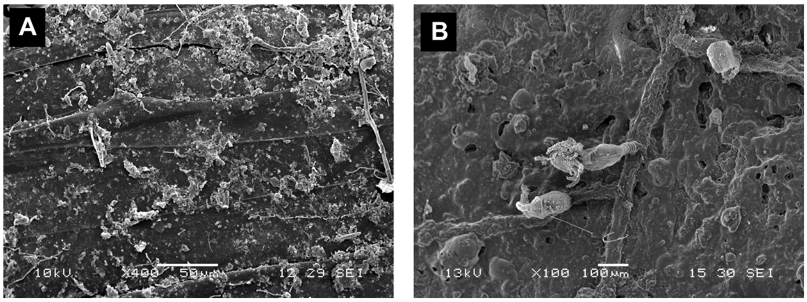

Figure 3 SEM micrographs of the flax/PLA panels after 3 months of immersion showing fouling by (A) micro-organisms and (B) hydrozoans.

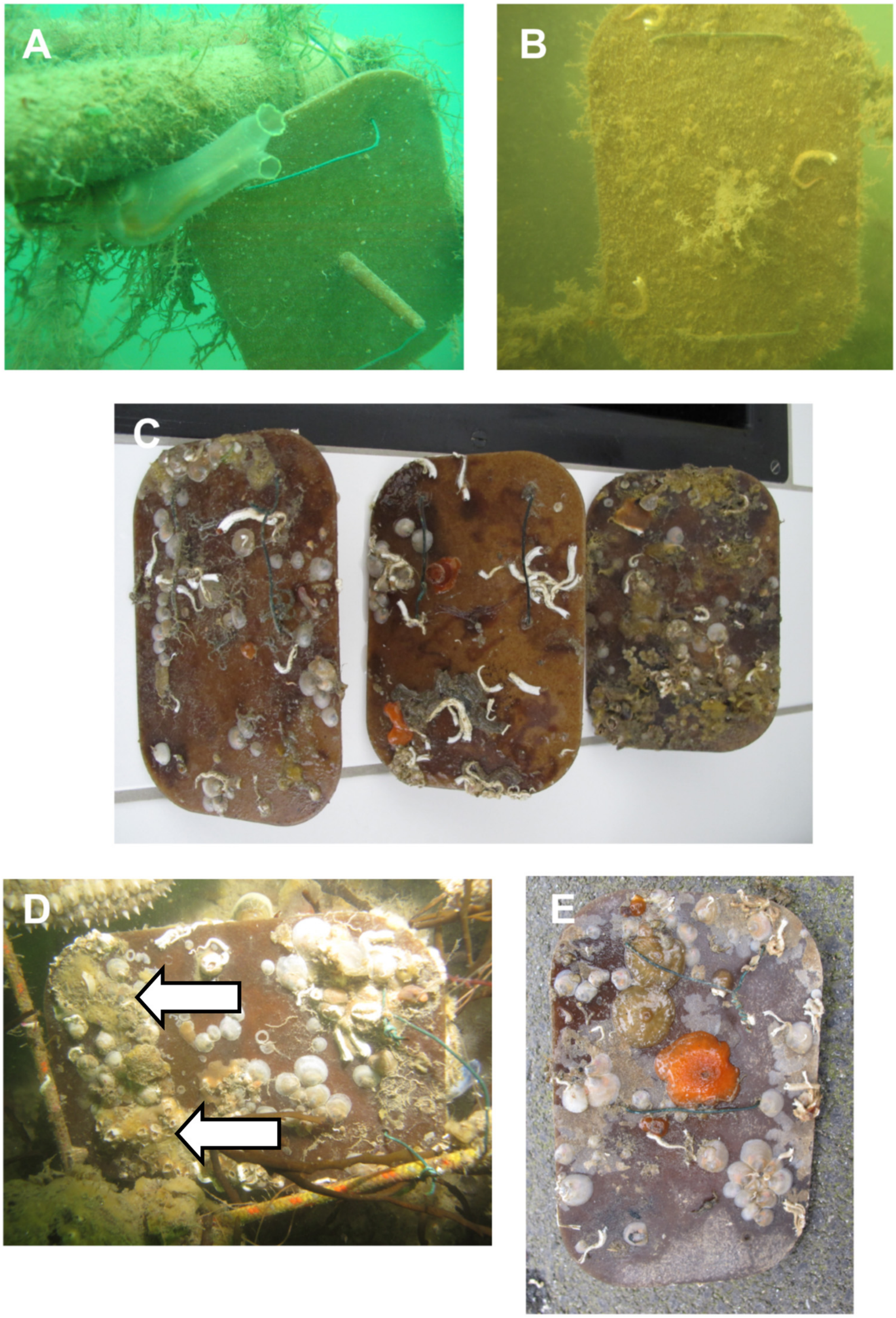

Figure 4 Fouling dominated by marine macro-organisms on PLA panels immersed into the Etel marina during (A) 90 days, (B) 6 months, (C) 2 years, (D) 3 years (showing barnacles with the marine sponge *Celtodoryx ciocalyptoides* (white arrows)), and (E) 6 years.

(Fig. 7). During 2012, the mean weight gain was significantly higher for specimens in nylon mesh bags than for those on PLA plates ($p < 0.05$). After two years (i.e. April 2013), *C. ciocalyptoides* specimens transplanted onto the PLA panels did not survive. Instead, both faces of the panels were covered of macrofoulers such as *Balanus* sp. and *Anomia* sp. This result suggests sponge cells did not find optimal conditions to adhere, survive and grow on flax/PLA biocomposite.

3.3. Transplantation efficiency of sponge

Measures of the weight of specimens let free in nylon mesh bags indicate no gain during the first year of transplantation while the increase in weight reached 100% the second year (Fig. 7). The composition in Metallic Trace Elements (MTEs) was quantified in transplanted specimens and compared with that from the natural site during the first three years (Fig. 8).

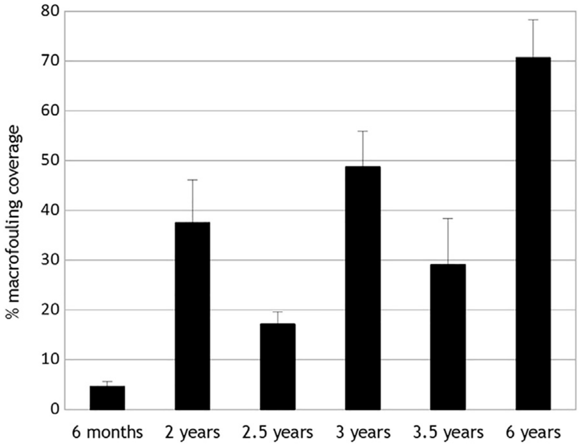

Figure 5 Percentage cover of marine organisms on PLA panels immersed into the Etel marina during the six-year study. Results are expressed as the mean ± SE of three replicates.

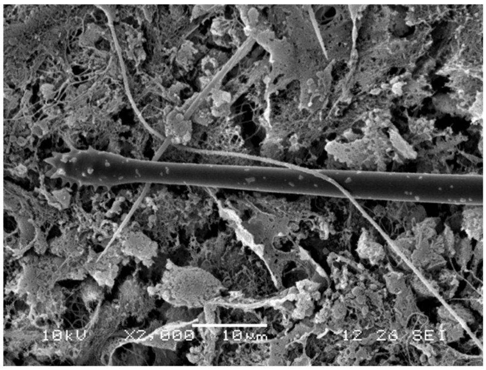

Figure 6 SEM micrograph of a flax/PLA panel showing a tylote constituting part of the skeleton of the species *Celtodoryx ciocalyptoides*.

Most of those MTEs, essential for sponge physiology, were at the same levels between transplanted and wild sponge specimens during the three first years of the study. Si levels remained particularly low in transplanted specimens but statistical analysis indicated no significant differences ($p > 0.05$) in comparison with wild ones. These results suggest transplantation in the Etel marina had no deleterious effects on the filtering activity of individuals which found favourable environmental conditions in the marina to survive and grow.

3.4. Sponge settlement on concrete substrate

After 5 months, fouling by barnacles *Balanus* sp., hydrozoans and rhodobionts covered approximately 75% of the surface of the scraped area on the concrete piling (Fig. 2b). In one year, the marine sponge *C. ciocalyptoides* was observed on the shells of barnacles, covering 13% of the initial area and competing with the hexacorallian *M. senile* and the tunicate *Diplosoma spongiforme* (Fig. 2c and d). At last, after two years, the scraped area got its original aspect with a density and thickness of *C. ciocalyptoides* similar to the other concrete pilings of the marina. This result confirms the sponge showed a rapid population growth during at least the first two years of our study.

4. Discussion

The colonization of artificial hard substrates by non-indigenous species is one of the major stumbling block in the development of coastal and marine infrastructure (Firth et al., 2016). In our study, we showed for the first time that a bio-based and biodegradable composite made of flax/PLA had promising anti-settlement and anti-attachment properties against the marine invasive sponge *C. ciocalyptoides*.

The recent observation of *C. ciocalyptoides* in the Etel marina close to shellfish farming activities suggests the possibility of its introduction through the Japanese Oyster *C. gigas* importation in Brittany during the 1970s. Once discharged in harbours, ports, and marinas sponge propagules found favourable conditions to establish on piers, pontoons, pilings, seawalls or buoys. Those artificial hard substrates are considered as providing new habitats facilitating the establishment, persistence and spread of non-indigenous and potentially invasive species (Glasby et al., 2007; Ruiz et al., 2009; Vaz-Pinto et al., 2014). We showed in this study that the colonization by *C. ciocalyptoides* on concrete pilings took place in a year by planktonic propagules (or competent sponge larvae) settlement and/or by spreading sponge colonies growing laterally. Finally, the marine invasive sponge *C. ciocalyptoides* was shown to cover up to 17.4% of the surface of concrete pilings of the Etel marina forming a mat of 5—

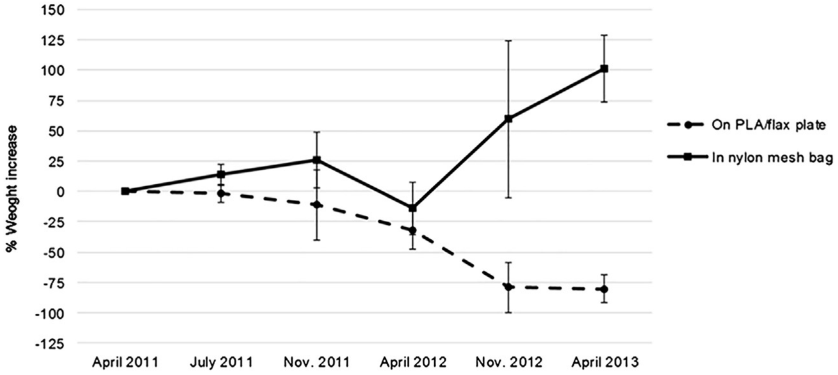

Figure 7 Weight variation of *Celtodoryx ciocalyptoides* transplants during the first two years. Results are expressed as the means ± SE of five replicates.

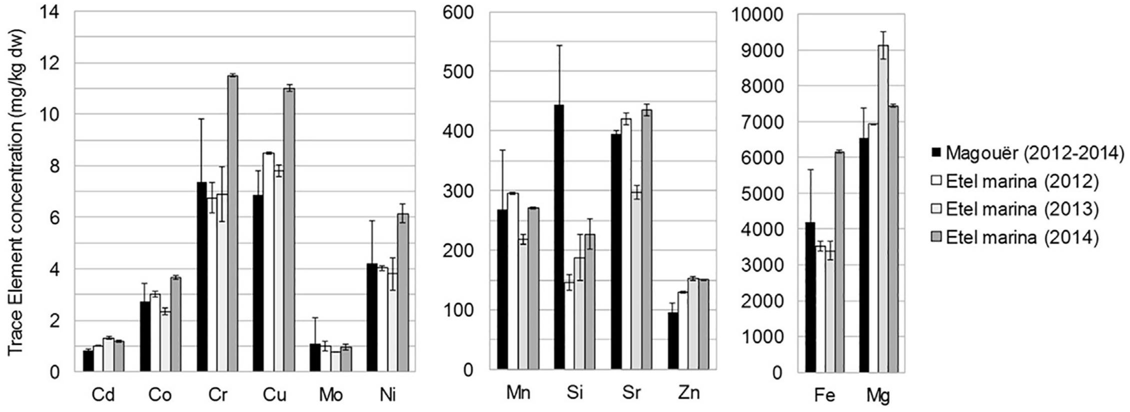

Figure 8 Elemental composition of the marine sponge *Celtodoryx ciocalyptoides*. Sponge specimens were transplanted into the Etel marina and their elemental composition was compared with that of wild specimens from the Magouër site during the first three years of the study (2012—2014). Results are expressed as the mean ± SE of three replicates.

6 cm of thickness from 5 to 9 m deep, between the mussel belt and the bottom of the marina (Gentric and Sauleau, 2016). Surprisingly, as shown in our study, fouling by marine organisms on flax/PLA biocomposite was delayed and reduced. After three years of immersion, fouling by successive macrofoulers such as barnacles, oysters and mussels covered half of the surface of the biocomposite. Specimens of *C. ciocalyptoides* were finally observed mainly on the shell of barnacles and in a lesser extent onto the flax/PLA panels. After six years of immersion, no invasive sponge cells were observed anymore. Further long-term studies are needed to assure sponge regression is due to PLA effect and not simply to a seasonal regression as already mentioned among non-indigenous species (Vaz-Pinto et al., 2014).

Concerning the anti-settlement and anti-attachment properties of the biocomposite, one can hypothesize that the bulk erosion of PLA and the release of lactic acid (LA) are incriminated in those phenomena. PLA degradation in compost and soil is already well documented (Jeon and Kim, 2013; Karjomaa et al., 1998; Nakamura et al., 2001; Sakai et al., 2001). Two steps are usually described in the degradation process of the polymer. Previous studies have shown that PLA degradation occurs mechanically and/or chemically depending on the water uptake, UV, pH, and temperature leading to the release of shorter oligomer chains and monomers of LA. In parallel, micro-organisms such as fungi and bacteria participate in the biofragmentation of the polymer by growing within the materials and provoking cracks. The second concomitant step is the enzymatic biodegradation by micro-organisms leading to carbon dioxide and/or methane and water. The biosynthesis of lipases, esterases, ureases and serine proteases (Lee et al., 2014; Matsuda et al., 2005; Sukkhum et al., 2009) cleaves the polymeric molecules into assimilable by-products. In contrast to terrestrial conditions, only few limited studies have been carried out on PLA biodegradation in the marine environment. PLA showed insignificant degradability in sea-water conditions at low temperature (18—21°C) even after immersion for 9 weeks (Zenkiewicz et al., 2012) or at 25°C for 10 weeks (Tsuji and Suzuyoshi, 2002). During tests conducted by the American Society of Testing and Materials (ASTM), the biodegradation of PLA was evaluated at 30°C. After 12 months of testing, results showed only 8% of the PLA sample biodegraded into carbon dioxide (CDRRR, 2012). In the Etel marina where our experiment was conducted, the seawater exceptionally reaches 20°C during summer in the first few metres. This low temperature may explain the relative slow degradation of PLA compared to soil or compost. Biopolymers reinforced with natural fibres provide biocomposites whose properties are comparable to those of traditional glass/polyester composites (Le Duigou et al., 2009). During a three-month immersion in seawater, the behaviour of PLA reinforced with 30% by weight of flax fibres showed a significant reduction in mechanical properties, in particular, a molecular weight decrease of almost 50% at 20°C. In comparison, the molecular weight of PLA decreased by 14% in the same conditions (Le Duigou et al., 2009). During ageing, water can diffuse by capillarity at the fibre/matrix interface and through the fibre itself (Le Duigou et al., 2009). This mechanism contributes to the hydrolysis of the matrix and may explain the long-term diffusion of small molecular weight PLA and LA from the core to the external layer of the PLA. As shown previously, a decrease in PLA molecular weight and increase in LA concentration directly depresses the attachment ratio of cypris larvae and inhibit the network formation of cement proteins involved in barnacle attachment (Ishimaru et al., 2012). Marine invertebrate larvae settlement is not completely understood but it seems that phototropism, biofilm formation and substrate topography play an important role in this process (Whalan and Webster, 2014; Whalan et al., 2015). In the perspective of a marine invasive species management, it will be of interest to understand the microbial biofilm formation at the interface between abiotic (i.e. flax/PLA biocomposite) and biotic (i.e. *C. ciocalyptoides* propagules) substrates.

To evaluate the anti-attachment properties of the biocomposite, sponge transplants were bound with an iron wire on PLA panels and their growth was compared with specimens let into nylon nets. To ascertain whether the transplantation itself had no direct effect on sponge growth and development, trace elements composition was measured as an indicator of the filter-feeding activity and compared with specimens living outside the marina. The roles of trace elements such as cadmium, copper, iron, silicon or zinc in sponge physiology seem to be essential (Mayzel et al., 2014). For example, silicon is required for the spiculogenesis in

Demospongiae. In our study, we showed transplants maintained a sufficient trace elements uptake during the first three years reflecting a normal pumping activity. The increasing levels of Ba, Cr, Cu and Ni measured in transplants are probably attributed to anthropogenic origins (urban runoff, antifouling biocides, etc.). For example, in marina, copper-based antifouling paints are largely applied onto ship hulls to limit the growth of fouling organisms. It's interesting to note that this sponge species accumulated Cu up to 11 mg kg^{-1} dry weight. In comparison, specimens living on the shore, outside the marina, accumulated in average 6.9 mg kg^{-1} dry weight. This copper tolerance confirms the inefficiency of copper-based antifouling paints to get rid of invasive species (McKenzie et al., 2012). In contrast, Si concentration was shown to be two times lower in transplanted samples. The Si uptake seemed, however, to increase by 15—20% each year suggesting sponge transplants adapted well to their new environment. Furthermore, weight gains indicated transplants let into mesh bags had a 100% increase in biomass after two years while transplants formerly bound on PLA panels did not survive. All these results indicated that the transplantation did not affect significantly sponge development and that the decline observed of our sponge transplants was due to the flax/PLA itself.

As harbours and marina are considered as the main entrance for invasive species, novel antifouling strategies are urgently needed. In this sense, an environmentally friendly approach using flax/PLA biocomposite was adopted to tackle the marine invasive sponge *C. ciocalyptoides*. In the present study, the occurrence of marine invasive sponge specimens was delayed for two years in comparison with concrete. In addition, fouling by marine invertebrates did not exceed 70% of the surface of flax/PLA panels even after 6 years of immersion. Artificial substrates such as pier pilings, boys, floats and pipes made of or coated with such biodegradable biopolymers may be useful in marina to slow or limit temporarily on their surfaces settlement by invasive species. Due to the increasing use of biopolymers for various application, many more studies on their biodegradation in marine environment are required.

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