

## Antifouling properties of chitosan coatings on plastic substrates

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## خصائص طلاء الشيتوزان المضاد للحشيف على ألواح البلاستيك

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**ABSTRACT.** In the current study, chitosan coatings were fabricated on plastic substrata and investigated for their anti-fouling activities. Scanning Electron Microscopy (SEM) and water contact angle measurement (WCA) of the fabricated chitosan films showed smooth and hydrophilic surface with WCA below 60°C. In the first experiment, chitosan coating on plastic substrate showed 88% reduction in settlement of bryozoan *Bugula neritina* larvae compared to the control after 3 hours incubation at dark conditions with no larval mortality. In the second experiment, the antimicrobial activity of chitosan was evaluated by coating plastic panels with the prepared chitosan solution and immersing the coated samples in seawater at controlled environmental conditions for two weeks. Biofilms scraped from immersed chitosan coated panels showed no bacteria after 1 week of immersion. After the second week of immersion, less than 1500 bacteria/mm<sup>2</sup> were observed on the chitosan-coated panels compared to more than 10<sup>5</sup> bacteria/mm<sup>2</sup> on uncoated ones. Thus, this study proved the efficiency of chitosan coatings against micro- and macro-fouling.

**KEYWORDS:** Chitosan; anti-larval activity; antifouling activity; bryozoan; biofilms.

**المستخلص:** في الدراسة الحالية، تم طلاء مادة الكيتوزان على ألواح بلاستيكية وبمحت إمكانية استخدامها كمادة مضادة للترسبات الحيوية البحرية. أظهرت تحاليل المسح بالمجهر الإلكتروني وقياس زاوية الاتصال بالمياه على أن أغشية الكيتوزان تتكون من سطح أملس ومحب للماء بزاوية اتصال مع الماء بدرجة أقل من 60°C. طلاء الكيتوزان على الأسطح البلاستيكية أظهر معدل انخفاض في تجمع يرقات *Bugula neritina* بنسبة 88% مقارنة بالأسطح غير المطلية بعد 3 ساعات من الحضنة في ظروف مظلمة. كما لم يتم ملاحظة أي وفيات في اليرقات خلال التجربة. تم تقييم النشاط المضاد للميكروبات لمادة الكيتوزان عن طريق غمر الألواح البلاستيكية المطلية بهذه المادة في كمية من مياه البحر مع وجود ظروف بيئية متحكم بها. تحليل الأغشية الحيوية المترسبة في الألواح المطلية بالكيتوزان والمغمورة في الماء أظهر عدم وجود أي بكتريا بعد أسبوع واحد من التجربة. بعد الأسبوع الثاني، تلاحظ وجود أقل من 1500 بكتريا/ملم على الألواح المطلية بالكيتوزان مقارنة بأكثر من 10<sup>5</sup> بكتريا/ملم على الألواح الغير مطلية. بالتالي، فإن هذه الدراسة تثبت كفاءة طلاء الكيتوزان ضد الترسبات الحيوية البحرية.

**الكلمات المفتاحية:** التراكم الحيوي على الأسطح المغمورة،

## Introduction

Natural and biodegradable biopolymers are currently receiving great interest to be used as alternative to petroleum polymers in terms of raw material supply and waste product reduction (Leceta et al. 2013). Chitosan is a biocompatible, biodegradable and bioactive biopolymer, which can be used in diverse industrial applications (Chatelet et al. 2001). It exhibits numerous interesting physicochemical and biological properties with various applications in water treatment, agriculture, biomedicine, food industry, and marine antifouling management (Cestari et al 2007; El-Sawy et al. 2010; Ramya et al. 2012; Luo & Wang 2013; Cai et al. 2016; Al-Naamani et al. 2017).

Chitosan possesses antimicrobial properties against

a number of bacteria, fungi and algae (Benhabiles et al. 2012; Lam & Diep 2015; Park et al. 2016). Antimicrobial properties of chitosan are due to the positively charged NH<sup>2+</sup> ions which interact with the negatively charged molecules in the microbial cell membranes leading to disruption of the cells (Coma et al. 2003; Alisashi & Aider 2012). The efficiency of the bactericidal effect of chitosan is dependent on various factors, such as micro-organism strain or species, environmental conditions (pH, temperature), molecular weight and concentration of chitosan as well as its physical state (solution or film) (Kong et al. 2010; Leceta et al. 2013).

According to the field of application, chitosan can be modified to form powder, flakes, gel beads, fibres or membranes and several other forms (Wang & Shen 2000; Pitakpoolsil & Hunsom 2014; Zhang et al. 2015). Chitosan films or membranes have found their ways in various industrial applications such as water desalination, as coating for food applications, wound dressing, and tissue engineering (Goy 2009; Sudha et al. 2015). Films can be easily prepared by dissolving chitosan powder or flakes in diluted acid solutions like acetic acid, and then casting the resulting solution on a flat surface or

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dipping or coating of any substrate, and finally allowing them to dry (Krajewska 2005; Goy 2009).

Biofouling is the undesirable attachment and growth of micro- (bacteria and diatoms) and macro-fouling (bryozoans, barnacles, mussels, etc.) organisms on man-made installations (Wahl 1989). Maritime industries spent billions of US dollars to prevent and control biofouling. Current ways of controlling biofouling include the use of toxic antifouling coatings that kill organisms and pollute the environment (Hellio & Yebra 2009). Thus, non-toxic antifouling solutions are urgently needed. Due to chitosan biodegradability, low toxicity to eukaryotes and environmental safety, chitosan films have been proposed as a green approach to prevent biofouling and as an alternative to toxic biocides (Pelletier et al. 2009). Chitosan was proven as a successful antifouling coating for membranes (Zhao et al. 2003; Zhou et al. 2010). A study by Yang et al. (2011) found that stainless steel functionalised with chitosan and hydroxyethylmethacrylate (HEMA) polymer reduced protein adsorption, bacterial adhesion, and exhibited antibacterial activity against *E. coli*. Chitosan films in laboratory experiments inhibited growth of fouling microorganisms, such as *Pseudomonas* and *Bacillus* (Machul et al. 2015; Zhou et al. 2013). A two months field study in northern Canadian waters was conducted with chitosan-based coatings (Pelletier et al. 2009). While the results of the study demonstrated promising antibacterial activity, the coating did not have any activity against algae. In our previous study, chitosan-zinc oxide nanocomposite coatings prevented growth of fouling diatoms and marine bacteria in laboratory and mesocosm experiments (Al Naamani et al. 2017). At the same time, the antifouling effect of chitosan films on larval settlement of major fouling species, like the bryozoan *Bugula neritina* (Dahms et al. 2004), has not been investigated.

The aims of this study were to: 1) fabricate chitosan coatings and characterise their physical and chemical properties, and 2) determine antifouling activity of chitosan coatings against micro- and macro-fouling organisms in laboratory experiments.

## Materials and Methods

### Preparation of chitosan solution

Two and a half grams of commercial chitosan powder of medium molecular weight with 110 cps viscosity and 95.6% deacetylation (Tru-Nutra Nutraceuticals LLC, India) were mixed with a volume of 100mL of 2% acetic acid (Sigma Aldrich, USA) to prepare 2.5% chitosan solution. The solution was kept under constant stirring for 24h at 25°C.

The viscous solution was coated on plastic substrates and allowed to dry for 24h at 26°C. The resulting coatings were characterized using FTIR spectrophotometry, scanning electron microscopy (SEM) and water contact

angle. The chitosan coatings were analysed directly using FTIR spectroscopic equipment (PerkinElmer, USA, Frontier™), in a spectral range from 4000 to 400 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup>. Surface morphologies of dry coatings were characterized using JEOL JSM-7200 (Japan) field emission scanning electron microscope (FE-SEM) working at 20 kV. The water contact angle (WCA) of coated substrates was measured using a Theta Lite Attension tensiometer (Biolin Scientific, Sweden) using a sessile drop technique to determine the films hydrophobicity (Al-Fori et al. 2014). A drop of 5µL water was placed in five different positions on each coating's surface. The right and left contact angles of each drop were measured and a mean water contact angle (WCA) was calculated from the resulting two values.

### Activity assessments of chitosan coatings on surfaces

#### Anti-larval activity assessment

To determine the anti-larval activity of chitosan, the prepared chitosan solution in acetic acid was coated on plastic panels (low density polyethylene, size 1cm<sup>2</sup>) and allowed to dry overnight. Three replicates of chitosan coated plastic panels were placed into a 24-well plate (Corning Costar, USA). Uncoated clean plastic panels were used as a control. Each well was filled with 1 mL seawater containing the bryozoan *Bugula neritina* larvae. Adult broodstocks of *B. neritina* were collected from pilings and floating rafts at Marina Bandar Rowdah. Larvae were obtained according to the method described by Bryan et al. (1997) and only newly (i.e., within 10min) released larvae were included in the bioassays.

Experiments were conducted under dark conditions by covering the 24-well plate with aluminium foil. In each well, the number of dead larvae, attached larvae and the total number of larvae were counted under a dissecting microscope (Zeiss, Germany, magnification 10X) after 1h, 2h and 3h. The percentages of larval mortality and larval settlement were calculated as follows (equations 1 and 2).

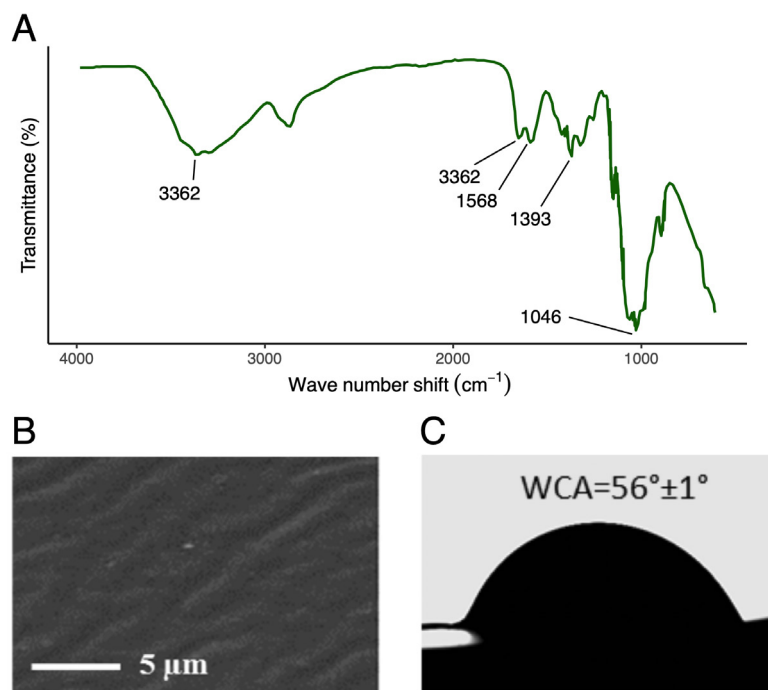
$$\text{Larval mortality (\%)} = (\text{NDL}) / (\text{TL}) \times 100 \quad \text{Eq.(1)}$$

$$\text{Larval settlement (\%)} = (\text{NSL}) / (\text{TL}) \times 100 \quad \text{Eq.(2)}$$

Where NDL is the number of dead larvae, TL is the total number of larvae and NSL is the number of settled larvae.

#### Anti-microfouling activity

To determine the antifouling activity of the chitosan in a small scale microcosm experiment, three different coatings were prepared: (a) 2.5% chitosan solution, (b) commercial two component non-toxic paint (Hempadur 45182, Hempel, Denmark) mixed with 2.5% chitosan solution at a ratio of 1:1 (v:v), (c) commercial two component non-toxic paint (Hempadur 45182, Hempel, Denmark). Plastic panels (Acrylic, 7.5 cm × 2.5 cm) were cleaned and both sides of the panels then painted with



**Figure 1.** (A) FTIR spectra of chitosan coating on plastic panels using 2.5% chitosan dissolved in 2% acetic acid, (B) SEM image of the chitosan coating, (C) Water contact angle of the chitosan coating.

a brush with each of the coatings. The coatings were allowed to dry for 24 hours at room temperature. Uncoated clean plastic panels were used as a control. Each panel was immersed vertically in a separate beaker containing seawater. Beakers were incubated at 26°C for 2 weeks. Each treatment and the control were replicated three times. After 1 week and 2 weeks, panels were removed. Biofilms from the whole area of the panel were scraped from the surface into Eppendorf tubes using a sterile scalpel. The remaining traces of the biofilm were rinsed with distilled water into the tube. Ten  $\mu\text{L}$  of the resulting biofilm suspension from each tube were added on a microscope glass slide and mixed with 10  $\mu\text{L}$  of SYBR Green 1 stain (SIGMA, Aldrich, USA) and incubated for 10 min. Finally, slides were analysed by an epifluorescence microscope. The number of bacteria in 20 randomly selected fields of view (Muthukrishnan et al. 2017) was counted and the total abundance of bacteria within 1  $\text{mm}^2$  was calculated.

## Results and Discussion

### Chitosan film characterization

The FTIR spectra of chitosan coatings (Figure 1a) showed a characteristic peak at 3362  $\text{cm}^{-1}$  for N–H and O–H stretching. Peaks corresponding to amide I and amide II were observed at 1662  $\text{cm}^{-1}$  and 1598  $\text{cm}^{-1}$ , respectively. The characteristic peak at 1046 is attributed to C–O stretching. The main absorption peaks of chitosan

films have been reported to be at 1650  $\text{cm}^{-1}$ , attributed to C=O stretching (amide I), 1558  $\text{cm}^{-1}$  attributed to N–H bending (amide II) and 1382  $\text{cm}^{-1}$  attributed to C–N stretching (amide III). The broad bands above 3000  $\text{cm}^{-1}$  assigned to O–H and N–H bonds while absorption peaks at 1050  $\text{cm}^{-1}$  were attributed to C–O stretching (Fernandez-Saiz et al. 2007; Leceta et al. 2013). The SEM images and WCA results showed a smooth and hydrophilic surface of chitosan coatings as shown in Figures 1b and 1c.

### Anti-larval activity of chitosan coating

*Bugula neritina* is a common fouling marine bryozoan with a short pelagic larval stage which can be found in warm temperate and subtropical waters worldwide (Ryland et al. 2011; Linneman et al. 2014). *Bugula* larvae have barrel-shaped bodies with their surfaces mostly covered with cilia that are referred to as a ciliated corona. Those cilia assist the larvae in swimming (Price et al. 2017).

The effect of chitosan coatings on the mortality and settlement of the bryozoan *Bugula neritina* larvae after 3 hours incubation in dark conditions is shown in Table 1. Effect of chitosan coating on *Bugula neritina* larval mortality and settlement after 1, 2, and 3 hours of incubation at dark conditions. Control is seawater.

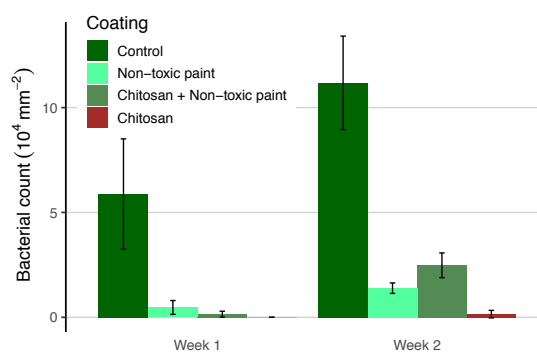
	% Larval mortality			% Larval settlement		
	1h	2h	3h	1h	2h	3h
Control	0	0	0	100±0	100±0	100±0
Chitosan	0	0	0	12±6.3	12±6.3	12±6.3

1. Results showed no larval mortality during the whole experiment. A 10-fold significant decrease in settlement of the larvae on chitosan films in comparison with the control was observed (Table 1). All larvae settled in the control and no changes in the settlement rate were observed during the incubation period. Overall, there was no difference in the larval settlement between 1h, 2h and 3h on chitosan film and the control. This can be explained by the fact that *B. neritina* has fast settling larvae that attach to the substratum within one hour (Bryan et al. 1997).

To our knowledge, this is the first study stating the effect of chitosan on the mortality and settlement of *B. neritina* larvae. Previously, Rasmussen et al. (2002) investigated the settlement of cyprids of *Balanus amphitrite* on chitosan gel crosslinked with glutaraldehyde. The authors observed a reduction in larval settlement to 35% when chitosan concentration was 2%. The settlement of *B. neritina* larvae on the surface of 8×10 cm plastic panels coated with low density polyethylene (LDPE), polypropylene (PP), polyvinyl chloride (PVC) and high density polyethylene (HDPE) was studied by Li et al. (2016). The authors reported higher larval settlement on the surface of panels coated with LDPE, PP, and PVC, compared to HDPE and glass panels after immersion in water for 4 days. The influences of organic films, such as chitosan, PP, PVC, etc., on larval settlement is not yet clear. It was suggested that physical, chemical and biological factors of the substratum, such as roughness, chemical properties of the substratum, presence of biofilms and other species, could affect larval settlement in the field (Clare et al. 1992; Faimali et al. 2004; Dobretsov et al. 2006; Qian et al. 2007; Li et al. 2016).

### Antimicrobial experiment

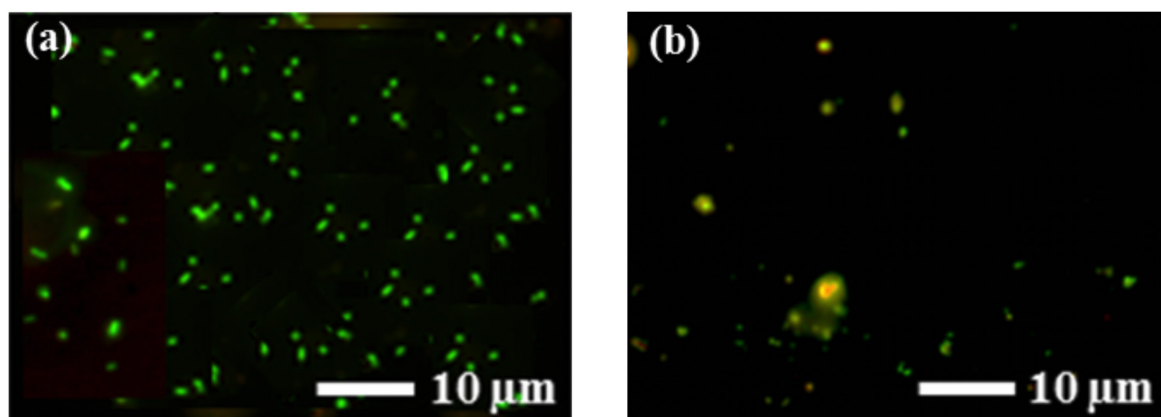
The experiment showed that the lowest bacterial abundances were found on chitosan coatings; no bacteria af-



**Figure 2.** Bacterial abundances on plastic panels coated with commercial non-toxic paint, commercial non-toxic paint mixed with 2.5% chitosan coating in ratio (1:1) and 2.5% chitosan coating. The control represents an uncoated panel.

ter 1 week and <1500 bacteria/mm<sup>2</sup> after 2 weeks of immersion were observed (Fig. 2). The highest densities of bacteria were found on the control substrata after 1 and 2 weeks of the experiment. The non-toxic antifouling paint was more effective than the mixture of non-toxic paint and chitosan (Fig. 2). Only a few algal cells were observed in the control samples. Comparatively, higher numbers of algal cells were observed on samples coated with non-toxic paint. However, no algal cells were observed on both the panels coated with a mixture of non-toxic paint and chitosan solution and that of only chitosan solution.

In the marine environment, the antifouling activity of chitosan was directly evaluated by Pelletier et al. (2009). They demonstrated antibacterial activity of 20% chitosan coating after 14 days of immersion in the sea, while 5% chitosan coating did not have any antifouling activity. Antibacterial activity of chitosan with concentration less than 2% chitosan was observed against *Ba-*



**Figure 3.** Bacterial cells as observed by epifluorescence microscopy at 1000X magnification in (a) biofilms scraped from the uncoated control plastic panels, and (b) biofilm scraped from panels coated with a mixture of chitosan and non-toxic paint. Cells were stained with CYBR Green I dye.

*cillus* sp., *Vibrio* and *Pseudomonas* sp., which are known to be involved in the biofouling process (Sekiguchi et al. 1994; Jumaa et al. 2002; No et al. 2002; Rasmussen & Østgaard 2003). It is well known that the cationic amine group in the chitosan molecule has a major role in its antimicrobial activity, as it forms electrostatic interactions with anionic group on the cell membrane of bacterial cells, which eventually lead to cell death (Rabea et al. 2003; Alisashi & Aider 2012). However, in our experiment chitosan charge has minor effect because of high pH of seawater (6.9-7.2) which neutralize most of the positive charges in chitosan's amino groups. Rasmussen & Østgaard (2003) suggested that surface energy was the crucial factor to prevent bacterial adhesion to the hydrophilic surface provided by chitosan at conditions of high pH. The anti-algal effect of chitosan was previously reported by Ravi Kumar (2000). However, no anti-algal activity of chitosan was observed by other researchers (Pelletier et al. 2009).

## Conclusion

In this experiment, chitosan solution was used to fabricate coatings characterised by FTIR, SEM and WCA. This chitosan solution was applied as a coating on acrylic plastic and antifouling effect against macro- and micro-fouling organisms was studied. The results of this study proved the effectiveness of chitosan coatings on the settlement inhibition of *Bugula neritina* compared to the control. The chitosan films also significantly reduced the density of fouling bacteria compared to non-toxic paint and control (no coating) when immersed in a natural seawater environment for two weeks. Those results suggest that chitosan is suitable to be used as coating component in order to prevent marine micro-and macro-fouling.

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