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COMPARING EFFECTS OF STARCH-BASED BIOPLASTICS AND CHITOSAN-STARCH BLENDS INGESTION IN SUPRALITTORAL AMPHIPOD *TALITRUS SALTATOR* (MONTAGU, 1808)

CONFRONTO DEGLI EFFETTI DELL'INGESTIONE DI BIOPLASTICHE SULLA SOPRAVVIVENZA DELL' ANFIPODE SOPRALITORALE TALITRUS SALTATOR *(MONTAGU, 1808)*

Abstract - *Marine supralittoral environments are particularly endangered by plastic and bioplastic accumulation. In this work, we assessed and compared the effects on survival rate related to ingestion of different kinds of starch-based bioplastics and chitosan-starch mixtures by supralittoral amphipod* Talitrus saltator*. Groups of adult individuals were fed with bioplastic/chitosan-starch blends presented as 10x10cm sheets, in controlled conditions. To measure palatability, sheets consumption was calculated using MatLab. Surviving individuals were counted after 14 or 28 days. Results show that chitosan is more palatable by sandhoppers when starch is present at a high percentage in the mixture, while there is no preference for starch-based bioplastics. In addition, after 14 days, survival rate drops drastically around 0% in chitosan experimental groups while, for starch-based bioplastic, survival rate is generally higher even after 28 days, depending on the type of bioplastic. Results highlight a negative effect on survival rate of sandhoppers, particularly regarding chitosan-starch blends.*

Keywords: *sandhoppers, survival rate, consumption, palatability, degradation*

Introduction - Recently, bioplastic research, development and production is gaining more and more attention due to the necessity to find alternatives to traditional petroleum-based plastics (Tong *et al.,* 2022). Though, bioplastic may be perceived as more "safe" by citizens, and this could lead to improper disposal of bioplastic waste in the environment. In this framework, marine and coastal environments are particularly impacted. We previously assessed the possibility of *Talitrus saltator* (Montagu, 1808), a key species in marine supralittoral environments, to ingest different types of starch-based bioplastic and of chitosan-starch blends even in absence of previous degradation or biofilm formation (Martellini *et al.*, 2023; Ugolini *et al*., 2024) In this study, we compared the palatability and the effects of ingestion of bioplastics and chitosan-starch blends on sandhoppers' survival rate.

Materials and methods - Adults of *Talitrus saltator* were collected on the beach of Regional Park of Migliarino, San Rossore, Massaciuccoli (Pisa, Italy). Amphipods were immediately transported to laboratory and placed in groups in plexiglass tanks in controlled conditions (artificial photoperiod L:D=12:12; T=25 ± 2 °C). For each group, the exclusive food source were represented as follows: paper and dry fish food as control; two different starch-based bioplastic, namely BIO1 and BIO2, obtained from well-known and widely used shopping bags brands (see Martellini *et al.,* 2023); and blends of chitosan-starch mixtures, prepared using the solvent casting method (Gupta *et al.*, 2022). Five different blends of chitosan starch-mixtures were prepared: chitosan 100%; chitosan 75% - starch 25%; chitosan 50% - starch 50%; chitosan 25% - starch 75 %; starch 100% (Ugolini *et al*., 2024). All samples were presented as 10x10 cm square sheets. For each treatment, the surface of sample sheets was measured after 7 days of experiment, taking digital pictures of the sheets and calculating the surface not eaten by amphipods in relation to the total surface using MATLAB (The MathWork, Inc.).

Furthermore, for each treatment, we calculated the number of individual still alive 14 days or 28 days after the start of the experiment (respectively, for groups fed with chitosan-starch mixtures or with starch-based bioplastic). In addition, fecal pellets were collected from tanks whitin two days from deposition. Analyses on starch-based bioplastic, chitosan-starch blends and related sandhopper's fecal pellets were carried-out by an IRAffinity-1S by SHIMADZU equipped with the Attenuated Total Reflectance (ATR) sampling accessory (MIRacleTM PIKE Technologies) and Gas cromatography-mass spectrometry (GC–MS) analysis (GC 7890A and MS 5975C Agilent Technologies), in the 4000 – 500 cm^{-1} range. Starch-based bioplastic and related fecal pellets were also analyzed by Proton Nuclear Magnetic Resonance (^{1}H-NMR) on a Bruker Avance 400 MHz instrument for further characterization. Statistical comparison between treatments was carried out using Mann-Whitney U test for consumption and G test for survival rate. Statistical significance was set at $p \leq 0.05$.

Results – Results related to consumption of sheets showed that BIO1 and BIO2 are both actively consumed with no significant differences and they both seem slightly preferred to control (BIO1 vs BIO2, p = 0.28; BIO2 vs Control, p = 0.065; BIO1 *vs* Control, p = 0.08, Mann-Whitney U test) (Martellini *et al.*, 2023). Chitosan-100% is practically intact after 7 days. Among chitosan-starch mixtures, chitosan 25% seems to be preferred by sandhoppers. The only significant comparison is between chitosan 75% and chitosan 25% (P = 0.021, Mann-Whitney U test). Starch 100% is always significant compared to the different chitosan/starch blends. When starch 100% is compared to control (paper and dry fish-food) no significant difference are higlighted (P = 0.365, Mann-Whitney U test) (Ugolini *et al*., 2024). Comparison among chitosan-starch blends and BIO1 and BIO2 shows that bioplastic is considered palatable and actively consumed by sandhopper (Fig. 1a). On the contrary, chitosan-starch blends are considered palatable only when starch is present at high percentage (chitosan 25%-starch 75%). (Fig. 1b)

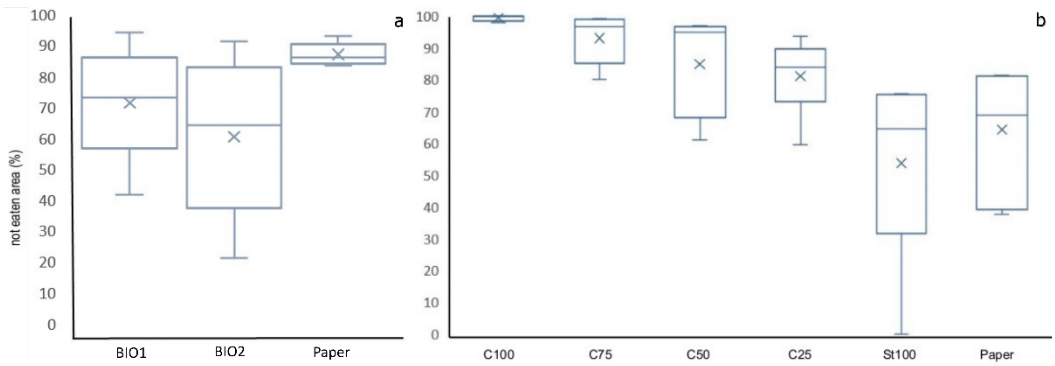

Fig. 1 - Boxplots showing uneaten area of paper (control) and experimental sample sheets. (a) BIO1, BIO2, starch-based bioplastic; (b) C100, Chitosan 100%; C75, Chitosan 75%, C50, Chitosan 50%; C25, Chitosan 25%; St100, Starch 100%; (modified from Martellini *et al*., 2023; Ugolini *et al*., 2024)

Boxplot che mostrano l'area non consumata della carta (controllo) e dei campioni sperimentali. (a) BIO1, BIO2, bioplastica a base di amido; (b) C100, Chitosano 100%; C75, Chitosano 75%; C50, Chitosano 50%; C25, Chitosano 25%; St100, Amido 100%; (modificato da Martellini et al.*, 2023; Ugolini* et al.*, 2024)*

Chemical analyses

Fourier Transformed Infrared Spectroscopy (FTIR) analysis were conducted on chitosan 50% and related fecal pellets, suggesting that only starch component is consumed by sandhoppers (Fig. 2), while, overlapping FTIR spectra of starch-based bioplastic and related fecal pellets, no significant differences are highlighted (not shown). The consumption of the starch component alone in chitosan-starch blends appears consistent with palatability findings.

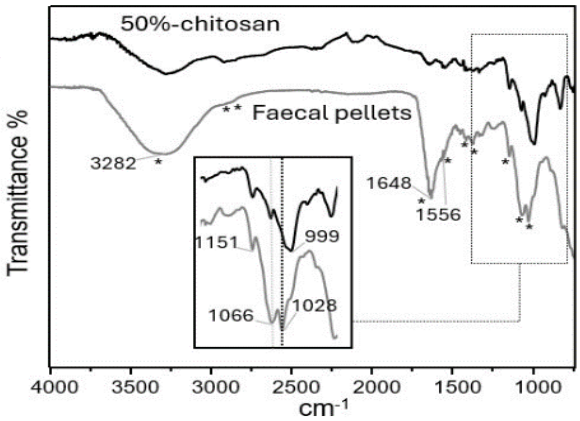

Fig. 2 - ATR-FTIR Spectra of chitosan 50% - starch 50% and of related fecal pellets collected after sandhopper's gut transit. The region highlighted represents C-O stretching associated to chitosan (at 999 cm^{-1} in Chitosan 50% and at 1028 cm^{-1}), that can be assumed as a diagnostic contribution of Chitosan in the spectra. (modified from Ugolini *et al.*, 2024)
Spettri ATR-FTIR di chitosano 50% - amido 50% e dei relativi pellet fecali raccolti dopo il transito intestinale dei talitri. La regione evidenziata rappresenta lo streching C-O associato al chitosano (a 999 cm^{-1} nel Chitosano 50% e a 1028 cm^{-1}), che può essere considerato un contributo diagnostico del Chitosano negli spettri. (modificato da Ugolini et al.*, 2024).*

NMR analysis conducted on starch-based bioplastic BIO1 highlights the decrease of the signal at 1.57 ppm after gut transit, wich is not encountered in BIO2. This could indicate the consumption of an aliphatic component of the bioplastic during sandhopper's gut transit in BIO1 (Martellini *et al.*, 2023). Summarizing, chemical analysis highlighted the possibility of degradation of material components both in starch-based bioplastics (at least in BIO1) and in chemical-starch mixtures (only for the starch component) by *T. saltator* during sandhopper's gut transit.

Survival rate

Comparing survival rate in starch-based bioplastic group, results indicates that BIO1 group survival rate (87%) is significantly higher than BIO2 (58%) (BIO1 vs BIO2: $G = 13.292$, $df = 1$, $p < 0.001$, G test) (Fig. 3a).

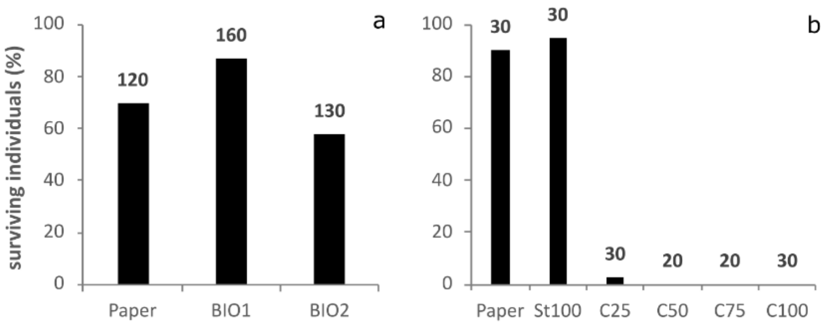

Fig. 3 - Survival rate of sandhoppers fed with different starch-based bioplastic (BIO1, BIO2) after 28 days (a), and Survival rate of sandhoppers fed with different kind chitosan-starch blends (b). Paper, control, St100, Starch 100%; C25, Chitosan 25%; C50, Chitosan 50%, C75, Chitosan 75%; C100, Chitosan 100% after 14 days (modified from Martellini *et al.*, 2023; Ugolini *et al.*, 2024)
Tasso di sopravvivenza dei talitri alimentati con diverse bioplastiche a base di amido (BIO1, BIO2) dopo 28 giorni (a) e tasso di sopravvivenza dei talitri alimentati con diverse miscele di chitosano e amido (b). Carta, controllo, St100, Amido 100%; C25, Chitosano 25%; C50, Chitosano 50%; C75, Chitosano 75%; C100, Chitosano 100% dopo 14 giorni (modificato da Martellini et al.*, 2023; Ugolini* et al.*, 2024).*

We hypothesize that the observed higher survival rate in BIO1 respect to BIO2 could be at least partially related to potential assimilation of lipid components of the bioplastic during gut transit (Martellini *et al.,* 2023). In chitosan-starch mixtures, after 14 days of experiment, survival rate of sandhoppers drop to zero in chitosan 100%, 75% and 50%, while, in chitosan 25%, stands at 3%. For starch 100% and control (paper) survival rates were higher (respectively, 95% and 90%), with no statiscal differences detected ($G = 0.314$, $df = 1$, p = ns, G test) (Fig. 3b).
Overall, different bioplastics have different effects on sandhoppers survival rate. However, sandhoppers fed with different starch-based bioplastic show higher survival rates compared to chitosan 100% and chitosan starch-blends.

Conclusions - Our results show that *Talitrus saltator* is able to feed both on starch-based bioplastic and chitosan-starch blends, although it seems clear that chitosan 100% seems to be not palatable by sandhoppers, and that palatability of chitosan-starch blends is related to the amount of chitosan contained in the mixture; noteworthy, it seems that only the starch component of chitosan-blends is subjected to digestion during sandhopper's gut transit.
These results are corroborated with survival rate data, showing that groups fed with chitosan-starch mixtures are characterized by a significantly higher mortality compared to starch 100% or control (paper) (Ugolini *et al*., 2024). We hypothesize that this could be related to a possibile intestinal obstruction effect of chitosan and/or to the note dietary fat absorption reduction property (Cheung *et al*., 2014). In addition, chitosan is known to possess antimicrobial effects (for a review, see: Ke *et al*., 2021), that could lead to a dysbiosis of sandhopper's gut microbiota. Regarding starch-based bioplastic, although no statistical differences in palatability are shown, sandhopper's survival rate is significantly higher in BIO1 compared to BIO2, despite these two bioplastic have very similar spectra and they are both attributable to polylactic-acid (Martellini *et al.*, 2023). In spite of this, survival rates related to the assumption of this two bioplastic are both higher than chitosan-starch mixtures. According to our results, we suggest to avoid the release of bioplastic and chitosan-starch mixtures in the environment. In particular, chitosan-starch mixtures seems to be particuraly harmful for sandhoppers. Nevertheless, a possibile role of *T. saltator* of degradation of starch-based bioplastic in the environment is highlighted.

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