Ulva and its components as potential stimulants in aquaculture feeds: chemosensory response of a valuable sea urchin species

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Abstract

Ulva is close to becoming popular due to its suitability as potential feedstock production and for food items. However, there is a general lack of studies on the aversion or acceptability of this green alga by marine organisms, in particular on its role as a chemoattractant and/or phagostimulant activity. Here we tested the effect of Ulva and other biochemicals as potential chemostimulating compounds for a valuable sea urchin species Paracentrotus lividus, selected as model species for our tests. Sea urchins’ chemical sensitivity was estimated by the analysis of movements of spines, pedicellariae, tube feet, and individual locomotion, using an innovative bioassay. Our results showed that all forms of Ulva (fresh, defrosted, and fragmented) resulted in an effective stimulus, evoking in sea urchins strong responses with robust activation of spines and tube feet, where the defrosted one was the most stimulating. Among the amino acids tested, glycine, alanine, and glutamine produced a significant response, highlighting for the latter a concentration-response relationship. Sea urchins responded to glucose, not to fructose and sucrose. Spirulina resulted as the most effective stimulus, acting in a dose-dependent manner. These results first indicate the role of Ulva and its most representative compounds as chemostimulant for such herbivore species. From an applied point of view, the presence of potential Ulva’s feed-related compounds, acting as chemoattractants (to reduce food searching time) and/or feeding stimulants (to stimulate ingestion), would improve the several applications of Ulva in the formulation of the feeds for sustainable aquaculture.

Introduction

The boosting of sustainable aquaculture for the aquatic plant will play a key element in the future of human and animal feeds production, agriculture biofertilizers, pharmaceuticals, cosmetics, emerging packaging, and waste treatment (FAO, 2021; Pereira, 2016). The margin on economic growth for this industry is mainly relevant in Europe, which contributes only 0.8% of world seaweed production (FAO, 2021).

On this challenge, the green algae belonging to the genus Ulva (Linnaeus, 1753) have been identified as one of the most suitable candidates for sustainable mariculture and are currently under the attention of the international scientific community generating many expectations (COST Action 20106; FAO 2021). The use of Ulva species may cover a wide range of products from human food, animal feed, and food ingredients to chemical constituents, and, on a broader view environmental benefits and ecosystem services (Campbell et al., 2019). However, citizen appreciation of Ulva and its usefulness in the food and feed industry still need to be further investigated and validated. In the context of new feed formulation which considers Ulva as a biomass source, one of the unknown aspects is whether this green alga is appreciated by the target farmed species. The main question regards biochemical compounds or traces elements of Ulva result in a chemical stimulation or chemoattraction towards the farmed species. Animals can perceive and respond to chemical cues in all environments, including aquatic habitats, where changes in behaviour after the detection of waterborne molecules have been extensively
documented for many species and in many behavioural contexts (Bargmann, 2006; Kamio and Derby, 2017; Mollo et al., 2017).

Sea urchins are slow-moving, broadcast-spawning marine invertebrates that rely on chemical signals to produce appropriate behavioural responses, such as avoidance of predators, spatial orientation, identification of suitable habitats, localization of potential food sources and conspecific mates (Lawrence, 2013). Pioneering studies have shown that sea urchins may trigger the activation of spines, tube feet, and pedicellariae in conspecifics (Snyder and Snyder, 1970; Campbell, 1983). For instance, it is known that Strongylocentrotus sp. is attracted by algae recognized as food (Vadas, 1977), while Lytechinus variegatus can orient to chemicals emanating from potential food resources over distance even under turbulent water flow conditions (Pisut, 2004). Sea urchins have been reported to respond to distant feeding stimuli with upstream orientation, using odour-guided rheotaxis for chemtrail navigation and odour source localization (Atema, 2012). In this respect, their slow speed may facilitate temporal sampling of chemical cues, and the widely distributed array of chemosensory organs may enhance spatial resolution (Weissburg, 2000).

Sea urchins also use chemicals in the fine-tuning of breeding aggregations and spawning synchrony strategies, to increase the probability of gamete encounter in animals with external fertilization (Mercier and Hamel 2009; Reuter and Levitan, 2010). Despite the broad chemical sensitivity of both larvae and adults of sea urchins, chemoreceptive organs have not been identified with certainty yet. Based on behavioural and histological studies, three main systems have been reported to respond to chemical cues: the “spine system”, the tube feet, and the pedicellariae, with responses ranging from simple, local reflex reactions of these systems to fully coordinated chemotaxis in which the whole animal moves toward or away from a stimulus source (Sloan and Campbell, 1982; Campbell et al., 2001). Sea urchin chemoreceptors belong to the family of the G-protein-coupled receptors (GPCRs) and their high number - up to several hundred - is comparable with that identified in many other animals, thus suggesting that sea urchins possess a sophisticated chemosensory system (Burke et al., 2006; Raible et al., 2006).

Here we considered the Mediterranean sea urchin Paracentrotus lividus as model species to investigate its chemosensory responses to a set of stimuli related to Ulva. The main questions regard the identification of the proper form in which these macroalgae should be used as the primary feed, and then, the identification of its biochemical compounds results in a chemical stimulation or chemoattraction towards the model species.

To face these questions, we carried out a series of trials that consider the following stimuli: fresh, defrosted, and freeze-dried Ulva, seawater conditioned with urchins fed with fresh Ulva, and seawater conditioned with faeces from urchins fed with fresh Ulva. Among biochemical compounds, we considered three sugars and six amino acids. Finally, the blue-green alga spirulina (Arthrospira platensis) was considered as a reference stimulus as it was tested in a previous investigation on the attractant response of sea urchins (Solari et al., 2021). Under the assumption that any of these substances could be stimulant and therefore could subsequently be considered as potentially a food source, we analyzed the
behavioral responses of sea urchins in terms of their locomotion, the movement of spines, pedicellariae, and tube feet by means of an animal bioassay.

**Materials & Methods**

**Animal collection and rearing conditions**

Wild specimens of *P. lividus* (*n*=100) measuring 30 mm in test diameter (corresponding to the third age class) were collected at a depth of about 5 m from the south coast of Sardinia (Italy). Prior to the experiments, the sea urchins were acclimated for two weeks in Plexiglas® tanks containing about 60 L of natural, aerated seawater (SW) at 20 ± 0.5 °C, 34‰ salinity, and a 12 h light/12 h dark photoperiodic regime. Animals were fed with the green alga *Ulva* sp. three times a week; uneaten food and/or faecal material were removed every 2 days along a partial (less than 10%) water exchange. The sea urchins were not fed for 48 h preceding the experiments to prevent any potential adaptation of their chemoreceptor neurons (CRNs) to chemical stimulation. All experiments were carried out in full accordance with the EU Directive 2010/63/EU.

**Stimuli and supply protocol**

The *Ulva* biomass was collected during the late spring season (May to June 2022) from a coastal lagoon located in the Gulf of Cagliari on the southern part of Sardinia, Italy (Santa Gilla 39° 13.760’N; 9° 4.761’E). The biomass collection was carried out using manual harvesting and it was washed with seawater to remove unwanted debris and stored in sterile plastic bags.

Fresh, defrosted and fragmented *Ulva* cultured water were considered as stimuli at 10 mg mL\(^{-1}\). Freeze-dried *Ulva* was prepared by suspending the relative amount of 10 mg/mL in seawater and filtered (Whatman Filter Paper, Sigma-Aldrich, Milan Italy). Moreover, faeces from *Ulva*-fed sea urchins (at the stock abundance of 4 specimens 4 litres\(^{-1}\) 40 g\(^{-1}\) of fresh biomass) were also considered as stimulants.

Among the amino acids, we considered the essential ones (methionine, valine) and non-essential ones (alanine, glycine, glutamine, serine) chosen as the most abundant (weight ratio) from the biochemical composition of *Ulva* (Prato et al., 2018). Among sugars, glucose, fructose, and sucrose were tested as stimuli. All amino acids and sugars were first dissolved in SW at 10\(^{-1}\) mol L\(^{-1}\) and were then stored frozen as stock solutions. On the day of the experiments, stock solutions were thawed and serially diluted in SW to be used at three different concentrations: 10\(^{-5}\), 10\(^{-3}\), and 10\(^{-1}\) mol L\(^{-1}\), and, in all cases, were supplied at increasing concentrations.

Spirulina, *Arthrospira platensis*, was prepared by suspending the finely hashed power in SW at 5 mg mL\(^{-1}\) and then accurately filtered (Whatman Filter Paper, Sigma-Aldrich, Milan Italy). Then, spirulina was serially diluted in SW to be used at 1, 0.1, and 0.01 mg mL\(^{-1}\).
Chemicals were obtained from Sigma-Aldrich (Milan, Italy), while freeze-dried spirulina was purchased from Livegreen Società Agricola (Italy).

Owing to the procedure adopted for the stimulus supply, actual concentrations to which sea urchins were exposed and to which they responded likely may have been less than those indicated, that is, the concentration diffusing from the inflow terminal to the tank containing the sea urchin tank. Therefore, we used a static system in such a way that each sea urchin was exposed to a Control condition (seawater at 20 ± 0.5 °C, 34‰ salinity, hereafter refers as SW) for 5 min and then to three different increasing concentrations of the same compound (four in the case of spirulina). During this stimulation sequence, the water was not replaced (i.e., stepwise stimulations were used, according to Solari et al., 2015; 2021).

Experiments were performed on 14-17 sea urchins for each tested compound and concentration. Each animal was tested with only one chemical at a time.

**Sea urchin bioassay**

Sea urchins were independently exposed to stimuli according to the procedure adopted by Solari et al. (2021). The experimental rack consisted of a small rectangular Plexiglas® tank containing about 350 mL of seawater which was connected to two different channels of a peristaltic pump (Gilson, Minipuls Evolution®) operating at a flow rate of 10 ml/min and thus ensuring a constant flow within the tank. The inflow and outflow terminals allow the seawater and chemical stimuli delivered into and removed from the tank. The outflow terminal was connected to a secondary tank for waste collection. At the beginning of each test, animals were immersed in the experimental tank and allowed to acclimatize until becoming motionless, typically within 15 min. Before the stimulus supply, the response of each animal to the same aliquot of seawater (blank control) was monitored for 5 min.

Stimuli were added to the tank for 1 min by switching the inflow terminal from seawater to a different reservoir and each sea urchin was allowed 4 min to respond, starting from the time the stimulus entered the experimental tank (typically 45 s after switching). This time frame was selected because of previous observations on dye diffusion in the experimental tank. Dye tests were also performed to verify the effectiveness of the perfusion/stimulation device. Trials were video-recorded for later analysis, using a color digital camera (Samsung SMX-F34, Korea) mounted above the experimental tank. Video recordings were analyzed by an independent observer blind to the experimental treatment.

The behavioral response was determined by measuring the movement rate of spines, tube feet, and the fully coordinated locomotion, if any, by which the whole animal moves toward or away from the outlet of the stimulus supply. At the end of the experiments, the sea urchins were returned to the holding tank.

**Detection of sea urchin movements**

All visible movements of the sea urchin spines and tube feet as well as the fully coordinated locomotory activity, when present, of the whole animal within the experimental tank, were captured by means of video recordings followed by a frame-to-frame computer analysis of the movements according to the procedure
adopted by Middleton et al. (2006) and Solari et al. (2021). Briefly, this approach produces an “urchinogram” in which the movements at several sites and levels on the same animal can be recorded and compared. The video recordings were converted to a resolution of 640×480 pixels, at 5 frames/sec (300 frames/min), so that each frame could account for the instantaneous “movement state” of the sea urchin at 200 msec intervals. Each video was analyzed using a custom program (AviLine, http://biolpc22.york.ac.uk/drosophila/ovary/) while the computer mouse was used to overlay lines on the video frames so that each line crossed the light/dark boundary between the animal (dark) and the background (clear). We adopted a grid with a total of 22 (13 vertical + 9 horizontal) evenly spaced lines, to cover the entire area of the experimental tank everywhere the animal moved. The mean square difference in light intensity (MSD) at each point of the lines in the grid between successive pairs of frames was plotted during the whole experiment. Therefore, the movements of the dark animal on the clear background generated changes in pixel intensity along the lines, which was used as an index of the movement rate of spines, tube feet, and locomotion of each sea urchin. Recording the MSD provides great sensitivity and good discrimination of movement, as it considers the change in every pixel along the line.

This analysis protocol recorded the displacement in the focus plane, but any movement in the vertical direction was not measured. Data were saved in a Microsoft Excel format and mean peak height and intervals between peaks were calculated. For each frame, the sum of values for all lines was calculated, to pick all movements of the spine, tube feet, and whole animal anywhere within the experimental tank. In this way, the amplitude of the sea urchin movements could be evaluated before and after the supply of the different stimuli.

Data analysis

Data are expressed as mean ± s.e. A paired t-test was used to evaluate the effect of each form of *Ulva* on the behavioral response of sea urchins, while the effects of the different concentrations of the other tested compounds were evaluated by one-way repeated measures ANOVA. Post-hoc comparisons were performed using Dunnett’s test to assess significant differences between each stimulus concentration and the relative blank (control) mean. When data did not conform to a normal distribution (Kolmogorov-Smirnov test for goodness of fit), Friedman's test was used for comparisons of repeated measures, followed by Dunn's post hoc test.

All statistical analyses were carried out by using the Prism program (GraphPad Software, San Diego, CA, USA). Differences were considered significant for $p \leq 0.05$.

Results

After being acclimatized in the experimental tank, the sea urchins became motionless and displayed only negligible, basal activity, consisting of slow oscillations of a few spines and limited movements of tube feet. Conversely, when exposed to a stimulating compound, the sea urchins started a typical response that was at first characterized by an increase in spine movements, coupled with a marked enhancement
in tube feet projectivity. This behaviour often culminated in a coordinated locomotory activity of the whole animal within the experimental tank. All these responses together were considered as an index of the chemical sensitivity of sea urchins toward a stimulus, according to what was previously reported by Campbell et al. (2001).

All forms of *Ulva* resulted in an effective stimulus, evoking in the sea urchins a robust activation of spines and tube feet (Fig. 1). In detail, compared to the Control, the increase in animal response ranged from 132.4 ± 6.4% following stimulation with fresh *Ulva* up to 169.7 ± 9.3% after supply of the defrosted one, which therefore resulted in the most stimulating form of the algae (MSD for the Control was 95201 ± 1894 in the case of fresh and 96978 ± 2339 for the defrosted *Ulva*, 100% of the response). As shown in the same figure, the sea urchins resulted also sensitive to the faeces from *Ulva*-fed animals, showing an increase in the movement rate of 147 ± 7.9% with respect to the Control (MSD was 93231 ± 2487).

The tested amino acids elicited different responses (Fig. 2). Non-essential amino acids, methionine, and valine failed to stimulate the sea urchins, regardless of the concentration used.

Conversely, among the essential amino acids, alanine and glycine were both stimulating with respect to the Control, but only at the highest tested concentration (10⁻¹ mol L⁻¹; Fig. 2). In fact, they increased the sea urchin response to 130.6 ± 8.3% and 160.7 ± 14.8%, respectively (MSD for the Control was 97679 ± 2301 in the case of alanine and 95684 ± 1994 for glycine). Glutamine too resulted in a stimulating amino acid, by increasing the sea urchin movement rate to 123.7 ± 6.1% and 142.5 ± 7.5%, compared to Control (MSD = 94002 ± 1826), when used at 10⁻³ and 10⁻¹ mol L⁻¹, respectively. Conversely, no significant changes in the movement rate were detected when the animals were presented with the essential amino acid serine, regardless of the concentration tested.

As for the sugars (Fig. 3), the sea urchins were completely insensitive to fructose, while they responded to its isomer glucose, which significantly enhanced the movement rate of the animals to 113.3 ± 4.3, 120.6 ± 6.3 and 136.1 ± 10.4% with respect to the Control (MSD = 95738 ± 3598) at the three concentration 10⁻⁵, 10⁻³ and 10⁻¹ mol L⁻¹, respectively. As shown in the same figure, the disaccharide sucrose never affected the movement rate of the sea urchins at any tested concentration. Finally, spirulina showed high stimulating effectiveness, affecting the sea urchins' motility in a dose-dependent manner (Fig. 4). In fact, even if the lowest dose (0.01 mg mL⁻¹) was ineffective compared to the Control (MSD = 102959 ± 3423), at 0.1 mg mL⁻¹ the blue-green algae evoked an increase in the movement rate of the animals to 133.5 ± 5.5% and further increased their response to 202.6 5 ± 9.1% when tested at 1 mg mL⁻¹. At the highest dose (5 mg mL⁻¹) the algae enhanced the animal movement rate up to 184.8 ± 7.6% with respect to Control, but this response did not statistically differ from that detected at 1 mg mL⁻¹.

**Discussion**

Based on the assumption that the overall movements of spines, pedicellariae, and tube feet could be classified as a behavioural indicator of chemical detection for a sea urchin species, we investigated such...
responses under the stimuli of Ulva and Ulva-related compounds. We used a bioassay already validated for sea urchins in response to chemical stimulation (Solari et al., 2021). Although we cannot affirm with certainty a direct correlation between the motor activation of sea urchins consequent to chemical stimulation and chemoattraction induced by Ulva, on the basis of our experiments, this green alga was definitely found to be an attractant and thus phagostimulant for the Mediterranean sea urchin P. lividus. Results have highlighted that all forms of Ulva, fresh, defrosted, fragmented Ulva cultured water, resulted in an effective stimulus, evoking in sea urchins strong responses with robust activation of spines and tube feet. Among Ulva tested, the defrosted one was the most stimulating. This result is particularly interesting because it highlights how the method of post-harvested storage of this macroalgae can affect the chemostimulatory properties and almost certainly its ingestion in our model species. A problem facing the use of wild Ulva in feed applications at our latitudes is that its harvesting is generally seasonal, and thus seaweed must therefore be preserved and stored to supply year-round production processes.

Speculatively, we can affirm that from the observations in our farming facility during the feeding processes of reared sea urchins fed with defrosted Ulva, the amount of uneaten defrosted Ulva is lower or absent in respect of the fresh ones (Secci et al., 2020), thus confirming its role not only as an attractant substratum but also as phagostimulant.

Freezing is one of the most popular means of long-term food storage, including macroalgae, allowing the preservation of the taste, texture, and nutritional value of foods better than any other preservation technology (Choi et al. 2012). Indeed, by transforming most of the liquid water into ice, freezing greatly slows the physical and biochemical changes involved in food deterioration and the growth and reproduction of spoilage microorganisms. In general, seaweed processed with a freeze/thaw cycle increases protein precipitation and doubles the total protein yield (Obluchinskaya & Daurtseva, 2020; Abdollahia et al., 2019; Kadam et al., 2015).

This mechanism could explain the strong response of sea urchins’ motility during our experiments a response similar to that we obtained also by using spirulina as a stimulant.

Although drying processes can alter the levels of certain natural nutrients in seaweed (Wong and Cheung 2001; Choi et al. 2012), this mechanism was not observed when the freeze-dried Ulva was used in our trials. On the other hand, when the freeze-dried spirulina was used, it resulted as the most effective stimulus, acting in a dose-dependent manner. The response to spirulina should be further investigated to identify if the strong activation of the tube feet, pedicellaria, and spines is an attractive or repulsive response. Recent studies confirmed several benefits of spirulina-enriched diets which improved gonadic growth and gamete production in P. lividus (Cirino et al., 2017) and enhanced the content of astaxanthin, a carotenoid with antioxidant properties and beneficial effects for various degenerative diseases, in the egg of Arbacia lixula (Galasso et al., 2018).

Of the other compounds tested, sea urchins responded to glucose, but not to its isomer fructose, while sucrose resulted ineffective. Among the amino acids tested, glycine and glutamine at the highest
dosages, and alanine, produced a significant response also highlighting a concentration-response relationship.

In conclusion, although it is well known that the behaviour of seaweed biomaterials is a key element in developing processing strategies that maximize the quality of products to be used as food and feed ingredients, the mechanisms of palatability for animal feed remain almost completely underexplored. New studies using two-choice treatments (Campbell et al., 2001) could help to identify the role of Ulva in the animal’s feeding choice (and the ingestion rate), in our case the sea urchin P. lividus, but also other reared species. The investigation of feed stimuli, in fact, can improve the optimization of rearing strategies by helping reduce the costs of the feed substrate in aquaculture.

Declarations

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Author contribution All of the experiments, data analysis, and manuscript preparation were conducted by P.S., P.A.; V.P.; Data curation, P.S., P.A., V.P., A.A. Writing – Original Draft Preparation, P.S., P.A.; Writing – Review & Editing, P.S., V.P., P.A., A.A.

All the authors reviewed and approved the manuscript.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information file.

Conflict of interest There are no conflicts of interest to declare in this work.

References


**Figures**
Figure 1

Normalized movement rate of sea urchins, recorded as mean square difference in light intensity (summed peak heights for each preparation during a 2-min stimulation) ± se (vertical bars), following supply of seawater (SW) conditioned with fresh, defrosted, freeze-dried (Dry) and fragmented Ulva and with faeces from Ulva-fed sea urchins, compared to SW (100% of response, dashed line).

*** and **** indicate significant differences for $p < 0.001$ and $p < 0.0001$, respectively (paired t-test) with respect to SW. Values are presented as means ± standard errors (vertical bars). The number of sea urchins tested for each stimulus is indicated in brackets.
Figure 2

Normalized movement rate of sea urchins, recorded as mean square difference in light intensity (summed peak heights for each preparation during a 2-min stimulation) ± se (vertical bars), following supply of the essential amino acids methionine and valine and the non-essential amino acids alanine, glycine, glutamine and serine, compared to seawater (SW = 100% of response, dashed line).
**, *** and **** indicate significant differences for $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively (Dunnett’s multiple comparison test subsequent to One-way ANOVA for valine and alanine; Dunn’s multiple comparison test subsequent to the Friedman test for methionine, glycine, glutamine and serine) with respect to SW. Values are presented as means ± standard errors (vertical bars). The number of sea urchins tested for each amino acid is indicated in brackets.

Figure 3

Normalized movement rate of sea urchins, recorded as mean square difference in light intensity (summed peak heights for each preparation during a 2-min stimulation) ± se (vertical bars), following supply of the monosaccharides fructose and glucose and the disaccharide sucrose, compared to seawater (SW = 100% of response, dashed line).

* indicates significant differences for $p < 0.05$ (Dunnett’s multiple comparison test subsequent to One-way ANOVA) with respect to SW. Values are presented as means ± standard errors (vertical bars). The number of sea urchins tested for each amino acid is indicated in brackets.
Figure 4

Normalized movement rate of sea urchins, recorded as mean square difference in light intensity (summed peak heights for each preparation during a 2-min stimulation) ± se (vertical bars), following supply of Spirulina, compared to seawater (SW = 100% of response, dashed line).
** and *** indicate significant differences for $p < 0.01$ and $p < 0.001$, respectively Dunnett’s multiple comparison test subsequent to One-way ANOVA) with respect to SW. Filled boxes indicate significant differences between a concentration and the next lower ($p < 0.05$; Tukey’s multiple comparison test subsequent to One-way ANOVA). Values are presented as means ± standard errors (vertical bars). Data were obtained from 15 sea urchins.