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# Turning an invasive alien species into a valuable biomass: Anaerobic digestion of *Rugulopteryx okamurae* after thermal and new developed low-cost mechanical pretreatments

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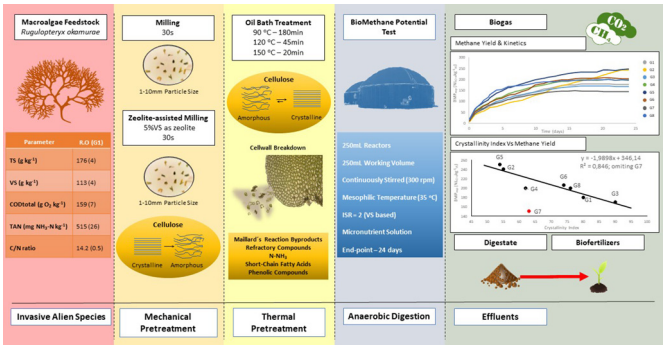
## HIGHLIGHTS

- AD is an efficient method to reduce the impact of the invasive alien *R. okamurae*.
- The newly developed mechanical pretreatment increased methane yield by 35 %.
- Cellulose crystallinity index showed a strong positive relationship with methane.
- The sole mechanical pretreated assay showed a two-substrate first-order kinetics.

## GRAPHICAL ABSTRACT

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## ABSTRACT

The invasive alien seaweed *Rugulopteryx okamurae* (*R.o.*) has spread quickly through the Mediterranean Sea causing an unprecedented ecological impact. A solution integrated into a circular economy model is needed in order to curb the negative effects of its presence. Anaerobic digestion (AD) is proposed as a feasible process able to transform biomass into renewable energy. Nevertheless, in order to improve the methane yield and surpass the drawbacks associated with AD processes, this research proposes a thermal pretreatment and a new developed method where the macroalgae is mechanically pretreated with zeolite. Chemical and microstructure characterization of the algal biomass after pretreatments involved scanning electron microscopy (SEM), X-ray powder diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR). The highest methane yields of 240 (28) and 250 (20) $NL_{CH4}\ kg^{-1}\ VS_{added}$ were obtained with the new mechanical pretreatment and the thermal pretreatment at 120 °C for 45 min without zeolite, achieving a 35 % improvement against the non-pretreated algae. A direct relationship between the crystallinity index of the samples and methane production was observed. The experimental data of methane production versus time were found to be in accordance with both first-order kinetic and Transference Function mathematical models.

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## 1. Introduction

In 2002, for the first time, the brown seaweed *Rugulopteryx okamurae* (*R.o.*) (Dictyotales, Ochrophyta) was observed in the Mediterranean Sea, nearby the Thau Lagoon (France) (Verlaque et al., 2009). Since then, it has widely spread through the Mediterranean Sea, being the Strait of Gibraltar the most affected area (El Aamri et al., 2018; Sempere-Valverde et al., 2021). Moreover, recently, it has been observed in the Azores archipelago located in the Atlantic Sea (Faria et al., 2022).

*R.o.* has spread through the Mediterranean Sea and beyond extremely quick and it has become a strong dominant species displacing local biota and causing an unprecedented ecological impact as well as being a source of negative impact on sea-dependant anthropogenic activities (El Aamri et al., 2018; García-Gómez et al., 2021; Sempere-Valverde et al., 2021). For the above-mentioned reasons, in 2020, *R.o.* has been included in the Spanish catalogue of invasive alien species (ministerial order TED/1126/2020 of the Ministry of Ecological Transition) and has attracted the focus of the scientific and industrial communities in order to understand the reasons of this unprecedented invasion and to evaluate its potential as profitable biomass.

Brown macroalgae are well known as biomass with high content on polysaccharides widely used in the food, nutraceutical, or pharmaceutical industries (Puri et al., 2022). Moreover, *R.o.* contains other high-value compounds with a wide range of bioactivities (e.g. inhibition of herbivores, antibiotic, anti-inflammatory, anti-viral, etc.) (Casal-Porras et al., 2021). Other uses have been proposed in order to valorise this biomass and thus reduce its impact. Among these, special attention has taken the development of bioplastics (Santana et al., 2022).

Anaerobic digestion (AD) is a biological process that occurs by the action of several anaerobic microorganism communities which transform organic matter into biogas, with a high content of methane (i.e. 60–70 %), and an effluent rich in several nutrients (e.g. minerals, bioavailable N, etc.) (Zamri et al., 2021). In this sense, AD is a promising process for circular economy models, since the produced biogas can be used as renewable energy and its nutrient-rich effluent can be used for soil applications (e.g. biofertilizer, soil bioremediation, etc.) (Fernández-Rodríguez et al., 2021; Zamri et al., 2021).

Several studies have been focused on the potential of different macroalgae as suitable biomass for AD (Saratale et al., 2018; Thompson et al., 2019). However, macroalgae as sole substrates present several drawbacks that do not allow for an efficient AD performance, i.e. low C/N ratio, which led to ammonium accumulation and the subsequent inhibition of the AD process, and the presence of non-degradable compounds (Saratale et al., 2018). In order to avoid these issues, other alternatives such as anaerobic co-digestion or the treatment of the algal biomass before the AD process have been proposed and evaluated (de la Lama-Calvente et al., 2021; Saratale et al., 2018; Suhartini et al., 2022).

Pretreatment of algal biomass before the AD process aims to break down and deconstruct the cell wall of the macroalgae by altering the structural compounds by physical, chemical, biological, or physicochemical means (Thompson et al., 2019). Among the physical pretreatments, the mechanical methods aim to reduce the particle size, while physicochemical pretreatments are focused on the use of temperature and/or pressure to breakdown not easily biodegradable compounds (Fernández-Rodríguez et al., 2020; Thompson et al., 2019). The effectiveness of thermal pretreatment has been already proven by industrial applications (Fang et al., 2011). Bordeleau and Droste (2011) showed that thermal pretreatments are very cost-effective alternatives compared to others, showing net costs per influent flow treated lower than those reported for chemical and ultrasound pretreatments and of the same order of magnitude as reported for microwave pretreatments. Literature shows a wide range of temperature, pressure and times used during the pretreatment of biomass, although, the most common range is 120 °C – 150 °C under pressure for up to an hour (Saratale et al., 2018).

On the other hand, mechanical pretreatments have been excluded to cutting down the biomass manually or by shredders, hammer mills, or any other type of conventional blender (Saratale et al., 2018). Although, the use of abrasives or inert solids during the milling process has been proved to be able to augment the degradability of cellulose (Oh and Kim, 1987), their effect on lignocellulosic biomass and the subsequent AD has not been studied any further. In this sense, zeolites are fine inert minerals that have been widely studied and inserted into AD systems due to their activity as ion-exchangers for nutrient removal, such as ammonia, as immobilizers of the anaerobic microbial communities or even as a biogas purifier (Guida et al., 2020; Kulawong et al., 2022). Moreover, zeolites are also well known for their catalyst capacity. However, to the best of our knowledge, zeolites have never been used as abrasives during the milling process of biomass destined to AD in order to enhance methane production, nor thermal pretreatments have been applied on *R.o.* before an AD process.

Therefore, the aims of this research were to assess the effect of a novel method using zeolite during the milling process of the *R.o.* and to study the effect of zeolite during thermal pretreatment at 90 °C, 120 °C, and 150 °C at heating times of 180, 45 and 20 min, respectively. Finally, pretreated biomass was used as a substrate for AD through biochemical methane potential (BMP) tests. Chemical and microstructural characterization of the macroalgal biomass after mechanical and thermal pretreatments was performed by instrumental techniques, i.e. scanning electron microscopy (SEM), X-ray powder diffraction (XRD) and Fourier-transform infrared spectroscopy (FTIR). Finally, kinetic modelling of the BMP assays was also carried out.

## 2. Materials and methods

### 2.1. Analytical methods

The anaerobic inoculum and the substrates (before and after each pretreatment) were analysed before the start-up of the BMP assays (Table 1). The resultant effluents obtained at the end of each experiment were also analysed (Table S3). Total solids (TS), volatile solids (VS) and mineral solids (MS) were determined in accordance with the standard method 2540B & 2540E (APHA, 2017). Total ammonia nitrogen (TAN) was analysed by distillation and titration following the standard method 4500-$NH_3$ (APHA, 2017). Total chemical oxygen demand ($COD_{total}$) was carried out as described by Raposo et al., 2008. Soluble chemical oxygen demand ($COD_{sol}$) was performed by the closed digestion and colorimetric standard method 5220D (APHA, 2017). pH and total alkalinity (TA) were carried out by using a pH meter model Crison 20 basic, and TA was analysed by titration to pH 4.3 and following the standard method 2320B (APHA, 2017).

Elemental analysis to determine the content of C, N and H was performed by a LECO CHNS-932 Elemental Analyzer (Leco Corporation, USA). Prior to the analysis, a representative sample of the macroalgae was brought to dryness by a lyophilization process. Analysis of soluble parameters were performed after sample centrifugation (Eppendorf, 9000 xg, 10 min) and filtration (Albet, 47 mm glass fiber filter).

Instrumental techniques for chemical and microstructural characterization included XRD, SEM and FTIR. XRD patterns were collected using Cu Ka radiation ($\lambda = 1.5406$ Å) in the 2θ range 5–50° with a step size of 0.02° and 3 s counting time per step. A Bruker D8 Discover diffractometer equipped with a high-resolution image plate detector was used. The crystallinity index was computed from the X-ray pattern by the Segal method (Segal et al., 1959). SEM analysis was performed on a FEI Teneo instrument in transmission mode. Infrared spectra were recorded on a JASCO FT/IR-480 plus spectrometer at room temperature, with disks prepared with 5 % of the samples in KBr. Spectra were collected by summing 24 scans at 4 $cm^{-1}$ resolution in the region 4000–400 $cm^{-1}$. Prior to the FTIR experiments, the samples were placed 48 h in a desiccator to remove moisture.

### 2.2. Anaerobic inoculum

The anaerobic inoculum used for the BMP tests was collected from a brewery wastewater treatment plant using an up-flow sludge blanket

**Table 1**
Main physicochemical parameters of the macroalga *R.o.* before and after pretreatments.

| | | | Pretreated at 90 °C, 180 min | | Pretreated at 120 °C, 45 min | | Pretreated at 150 °C, 20 min | |
|---|---|---|---|---|---|---|---|---|
| Parameter[a] | *R.o* (G1) | *R.o.* & Zeolite (G2) | *R.o.* (G3) | *R.o.* & Zeolite (G4) | *R.o.* (G5) | *R.o.* & Zeolite (G6) | *R.o.* (G7) | *R.o.* & Zeolite (G8) |
| TS (g $kg^{-1}$) | 176 (4)$^{1}$ | 162 (4)$^{2}$ | 186 (3)$^{3}$ | 209 (5)$^{4}$ | 154 (3)$^{5}$ | 218 (5)$^{6}$ | 209 (4)$^{4}$ | 171 (5)$^{1}$ |
| VS (g $kg^{-1}$) | 113 (4)$^{1}$ | 103 (6)$^{2}$ | 133 (3)$^{3}$ | 137 (8)$^{3}$ | 100 (3)$^{2}$ | 136 (2)$^{3}$ | 137 (3)$^{2}$ | 101 (3)$^{3}$ |
| VS/TS ratio | 0.64$^{1}$ | 0.64$^{1}$ | 0.72$^{2}$ | 0.66$^{1}$ | 0.65$^{1}$ | 0.63$^{1}$ | 0.66$^{1}$ | 0.59$^{3}$ |
| CODtotal (g $O_2$ $kg^{-1}$) | 159 (7)$^{1}$ | 150 (10)$^{2}$ | 190 (16)$^{3}$ | 190 (16)$^{2}$ | 140 (11)$^{3}$ | 184 (9)$^{2}$ | 190 (15)$^{3}$ | 150 (12)$^{4}$ |
| TAN (mg $NH_3$-N $kg^{-1}$) | 520 (26)$^{1}$ | 490 (25)$^{2}$ | 470 (34)$^{2}$ | 470 (32)$^{2}$ | 470 (32)$^{2}$ | 450 (28)$^{2}$ | 480 (18)$^{2}$ | 420 (25)$^{3}$ |
| C (%)[b] | 36.0 (0.4)$^{1}$ | 33 (2)$^{2}$ | 37 (1)$^{1}$ | 34.9 (0.4)$^{2}$ | 35.0 (0.6)$^{2}$ | 34 (1)$^{2}$ | 32.8 (0.4)$^{2}$ | 32.0 (1.1)$^{3}$ |
| N (%)[b] | 2.5 (0.1)$^{1}$ | 2.5 (0.3)$^{1}$ | 2.64 (0.08)$^{1}$ | 2.46 (0.05)$^{1}$ | 2.39 (0.07)$^{1}$ | 2.24 (0.08)$^{1}$ | 2.17 (0.07)$^{2}$ | 2.07 (0.08)$^{2}$ |
| H (%)[b] | 5.1 (0.1)$^{1}$ | 4.2 (0.2)$^{2}$ | 4.7 (0.1)$^{3}$ | 4.69 (0.08)$^{3}$ | 4.73 (0.08)$^{3}$ | 4.6 (0.1)$^{3}$ | 4.4 (0.1)$^{2}$ | 4.3 (0.1)$^{2}$ |
| O (%)[b] | 20.7 (0.6)$^{1}$ | 23 (2)$^{2}$ | 27 (1)$^{1}$ | 23.5 (0.5)$^{1}$ | 23.1 (0.7)$^{1}$ | 21 (1)$^{2}$ | 26.1 (0.6)$^{1}$ | 20 (1)$^{2}$ |
| C/N ratio | 14.2 (0.5)$^{1}$ | 13 (1)$^{2}$ | 14.02 (0.05)$^{1}$ | 15.1 (0.4)$^{1}$ | 14.6 (0.2)$^{3}$ | 14.1 (0.3)$^{1}$ | 15.1 (0.4)$^{3}$ | 15.2 (0.1)$^{3}$ |

[a] Values represent mean (standard deviation). Different superscripted numbers in the same row indicate values are significantly different ($\alpha = 0.05$).
[b] Based on dry matter.

reactor located in Seville, Spain. This inoculum was selected due to its high methanogenic activity, which was confirmed by positive controls during the BMP tests. The inoculum was immediately placed in a water bath at 35 (2) ºC for 36 h before its use in order to reduce the background methane production.

The main characterization parameters of this inoculum were as follow: TS, 50.2 (0.8) g $kg^{-1}$; VS, 37.9 (0.8) g $kg^{-1}$; $COD_{total}$, 53 (2) g $O_2$ $kg^{-1}$; TAN, 253 (13) mg $NH_3$-N $kg^{-1}$; pH, 7.53 and TA, 2799 (62) mg $CaCO_3$ $L^{-1}$.

### 2.3. Macroalgae substrate

The invasive seaweed *R.o.* was provided by the Laboratory of Marine Biology of the University of Seville. The macroalgae was collected from Tarifa Island (36º05'20.38"N,5º48'45.34"O), washed in-situ with sea water to remove debris and stored at −20 °C until further use.

Before any further use, the seaweed was unfrozen at 4 °C for 2 h and any observable debris in plain sight was removed.

### 2.4. Zeolite

The natural zeolite used in this study was donated by the Laboratory of Zeolites of the University of Havana. Zeolites were obtained from the Tasajeras deposit (Villa Clara, Cuba), and then subjected to a mineral benefit method resulting in a mixture of clinoptilolite (70 %), mordenite (5 %), anorthite (15 %), and quartz (10 %). The chemical composition of the material in oxide form and using water as balance was $SiO_2$ 66.50 %, $Al_2O_3$ 11.30 %, CaO 4.00 %, $Na_2O$ 1.95 %, $K_2O$ 1.12 %, MgO 0.65 %, and $Fe_2O_3$ 1.80 %. The zeolite was grounded and sieved in the 30–90 μm particle size range.

### 2.5. Experimental procedure

#### 2.5.1. Pretreatments

Two different pretreatments and combinations of both were investigated; the effect of zeolite during milling and the effect of temperature during thermal pretreatment. The milling process was assessed preliminary in order to determine the shorten time needed to reduce the particle size of the macroalgae to 1-10 mm by a commercial blender (120 W, Aromatic FP905S, Taurus, Spain). It was concluded that for both milling processes (i.e. with and without zeolite) the adequate time was 30s per 10 g of biomass.

For the pretreatment with zeolite, 5 % of zeolite (VS based) was mixed with the macroalgae before milling. Thermal pretreatment was performed by adding 40 g of substrate into 250 mL tight-closed Pyrex glass bottle and heated in an oil bath (Precis-bat 4200 W, Selecta, Spain). The investigated temperatures were 90 °C, 120 °C and 150 °C, and the duration of each pretreatment was 180 min, 45 min and 20 min, respectively. These temperatures and times were selected based on literature. Thermal pretreatments are widely used and studied, being the most common range between 120 °C and 150 °C for up to an hour (Saratale et al., 2018; Fang et al., 2011). In the present study, a low temperature (90 °C) was also included and the time for each temperature was chosen based on a preliminary energy evaluation so each combination would have a similar cost. Besides, it is well known that high temperatures and times have a negative impact on AD performance due to the production of refractory materials and the release of toxic compounds. Finally, the combination of both pretreatments was carried out by milling the macroalgae with zeolite and continuing with the thermal pretreatment as described above. For further clarity and in order to help the reading and understanding of the manuscript, the Table S1 is provided in the Supplementary Material document as a resume of the treatments carried out in each assay.

Table 1 shows the main physicochemical characteristics of the substrates after each pretreatment. The samples were labeled as follows: G1: raw *R.o.*; G2: mechanically pretreated *R.o.* with zeolite; G3: thermally pretreated *R.o.* without zeolite at 90 °C, 180 min; G4: thermally pretreated *R.o.* with zeolite at 90 °C, 180 min; G5: thermally pretreated *R.o.* without zeolite at 120 °C, 45 min; G6: thermally pretreated *R.o.* with zeolite at 120 °C, 45 min; G7: thermally pretreated *R.o.* without zeolite at 150 °C, 20 min and G8: thermally pretreated *R.o.* with zeolite at 150 °C, 20 min.

#### 2.5.2. Biochemical methane potential (BMP) tests

The BMP tests were set up by following the indications described by Holliger et al. (2016). All tests, including blanks and positive control, were carried out in triplicates. Blanks were prepared following the same indications described below for samples but with no substrate, and, as a positive control, microcrystalline cellulose (Avicel® PH-101, Fluka) was used.

The BMP tests were performed at mesophilic temperature (35 °C) with an inoculum to substrate ratio (ISR) of 2 (VS based). 250 mL reactors were used and filled with inoculum, substrate, 0.1 % (*v*/v) micronutrient solution, as described in Fernández-Rodríguez et al. (2020), and distilled water up to a working volume of 250 mL. Reactors were placed into a water bath at a controlled temperature and flushed with nitrogen gas at the beginning of the experiment.

The produced biogas was passed through a 2 N NaOH solution and the remaining gas, which was measured volumetrically, was assumed to be methane as supported by Casallas-Ojeda et al. (2022) and is widely reported in the literature (Carrère et al., 2008; Rincón et al., 2013).

The experiment was assumed to be finished when the accumulated volume of methane was <1 % for three consecutive days, this period was c.a. 24 d. The accumulated methane yield was corrected by subtracting the production of the blanks and expressed under standard temperature and pressure conditions (273.15 K and 101.33 kPa).

### 2.6. Data analysis

#### 2.6.1. Biodegradability

The biodegradability of each substrate was calculated as proposed by Nielfa et al. (2015). The theoretical methane production was calculated

by both $COD_{total}$ and elemental composition (CHON) analysis. Results obtained and discussion about biodegradability is included in the Section 2 of the Supplementary Material document.

*2.6.2. Kinetic models*

In order to study the process kinetics and estimate the process performance in the anaerobic digestion of the different substrates studied three models were considered.

*2.6.2.1. First-order kinetic model.* The following first-order kinetic model was used (Rincón et al., 2013; de la Lama-Calvente et al., 2021):

$$G = G_m \cdot [1 - \exp(-k \cdot t)] \quad (1)$$

where:

$G$ is the cumulative specific methane production ($NL_{CH4}\ kg^{-1}\ VS_{added}$)
$G_m$ is the ultimate methane production ($NL_{CH4}\ kg^{-1}\ VS_{added}$)
$k$ is the specific rate constant ($day^{-1}$)
$t$ is the digestion time (day).

*2.6.2.2. Transference function model.* The Transference Function (TF) model was also used to fit the experimental data of methane production during BMP tests (Fernández-Rodríguez et al., 2020; de la Lama-Calvente et al., 2021). The TF model is given by the following expression:

$$B = B_{max} * \left(1 - exp\left[-\frac{R_{max}(t-\lambda)}{B_{max}}\right]\right) \quad (2)$$

where:

$B$ ($NL_{CH4}\ kg^{-1}\ VS_{added}$) is the cumulative specific methane production
$B_{max}$ ($NL_{CH4}\ kg^{-1}\ VS_{added}$) is the ultimate methane production
$R_{max}$ is the maximum methane production rate ($NL_{CH4}\ kg^{-1}\ VS_{added}\ d^{-1}$)
$t$ (days) is the digestion time
$\lambda$ (days) is the lag time.

*2.6.2.3. Two-substrates kinetic model.* The methane production data from the BMP test corresponding to the trial with zeolite without thermal pretreatment was also modeled by a two-substrates kinetic model. In this model, the mentioned substrate assessed was composed of a rapidly biodegradable substrate type and a slowly biodegradable substrate type; *R.o.* biomass treated with zeolite during milling was degraded according to the rate

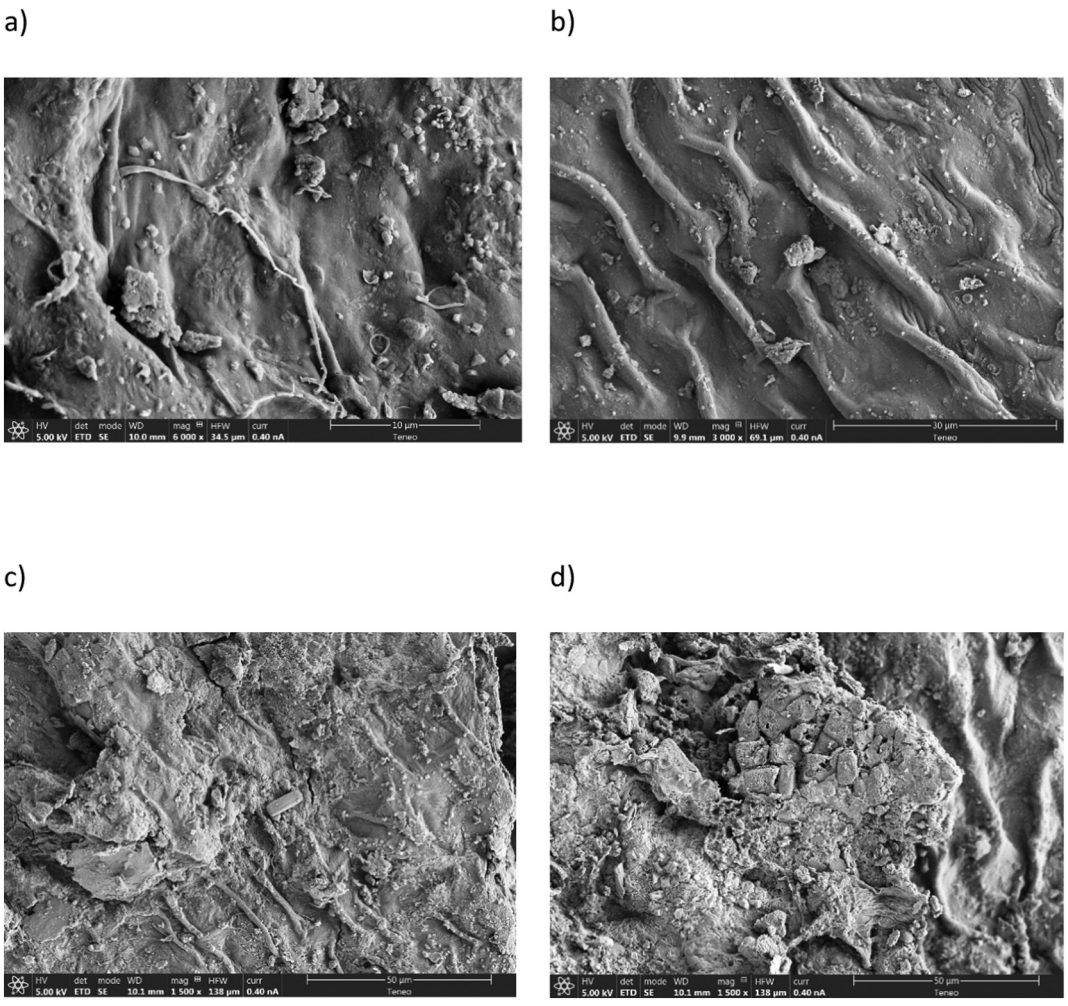


**Fig. 1.** SEM images of raw *R.o.* (G1) (a), mechanically pretreated *R.o.* with zeolite (G2) (b), thermally (150 °C, 20 min) pretreated *R.o.* without zeolite (G7) (c) and thermally (150 °C, 20 min) pretreated *R.o.* with zeolite (G8) (d).

constant of the respective fractions (Bai et al., 2016; Scarcelli et al., 2020). This two-substrates kinetic model is given by Eq. (3):

$$B = B_{rapid}\left[1- \exp\left(-k_{rapid}{}^{*}t\right)\right] + B_{slow}[1- \exp\left(-k_{slow}{}^{*}t\right)]) \quad (3)$$

where:

$B$ ($NL_{CH4}\ kg^{-1}\ VS_{added}$) is the methane production
$t$ (days) is the digestion time
$B_{rapid}$, $B_{slow}$ ($NL_{CH4}\ kg^{-1}\ VS_{added}$) correspond to the maximum methane yields of each fraction
$k_{rapid}$ and $k_{slow}$ ($d^{-1}$) are the kinetic coefficients of each fraction.

### 2.7. Statistical analysis

All the analysis and results were at least carried out in triplicates and values are given by means (standard deviation). For single comparisons, a two-tale Student's *t*-test was carried out, while for multiple comparisons, the one-way analysis of variance (ANOVA) was the selected method. For the purposes of data discussion, a $p < 0.05$ value was accepted as statistically significant.

## 3. Results and discussion

### 3.1. Chemical and microstructural characterization of the samples after pretreatments

SEM analysis was carried out in *R.o.* before and after mechanical and thermal pretreatments to study their effects on the surface texture. As shown in Fig. 1a, untreated *R.o.* presented a uniform and smooth surface with typical folds as found in other brown algae (Bogolitsyn et al., 2020) and with an intact surface structure containing minor impurities. However, after the mechanical and thermal pretreatments the surface of the algae exhibited micro-pores and small pieces of debris (Fig. 1b-d), which is in accordance with the findings of Ding et al. (2020) who reported damages in the structure of the brown seaweed *Laminaria digitata* after thermal pretreatment (140 °C for 20 min), concluding that more areas were available for glucoamylase to contact, enhancing the biogas production.

The XRD diffraction patterns of untreated and pretreated *R.o.* samples are shown in Fig. 2. The peaks observed in the untreated *R.o.* are associated to the crystalline structure of cellulose I, and correspond well to the simulated diffractogram from the crystal structure. After mechanical and thermal pretreatments, the cellulose I form was preserved in all samples. However, an impact on the crystallinity of the samples was observed, as evidenced by the deteriorated signal/background ratio. The values of the crystallinity indexes from G1 to G8 (80, 55, 90, 62, 54, 74, 63 and 76, respectively) evidenced the structural damage by the applied pretreatments, despite the milling lasted only 30 s. On the other hand, the relative lowering of the intensity of the diffraction peaks associated to cellulose unmasked the presence of other mineral compounds, such as silica (e.g. at $2\theta = 26.6^{\circ}$ in G4) and calcite (e.g. at $2\theta = 29.5^{\circ}$ in G4, G5 and G6), among others, which could be due to the high percentages of MS on the studied biomasses (28.4 % – 40.9 % d.m.), as was also reported in other brown algae (Medaković et al., 1995; Gómez and Westermier, 1995). SEM images also showed the presence of diatom skeletons (regular spot seen in the center of Fig. 1c) which are made of very pure silica and other carbonated compounds, and could be partially responsible of the high MS content.

Comparing the crystallinity of cellulose after thermal treatments at 120 °C and 150 °C, it was apparent that the thermal effect was enough to generate amorphization of the cellulose in *R.o.*, while at 90 °C a higher crystallinity was observed. This suggested that at low temperatures the reversible reordering of the cellulose chains was more significant than the

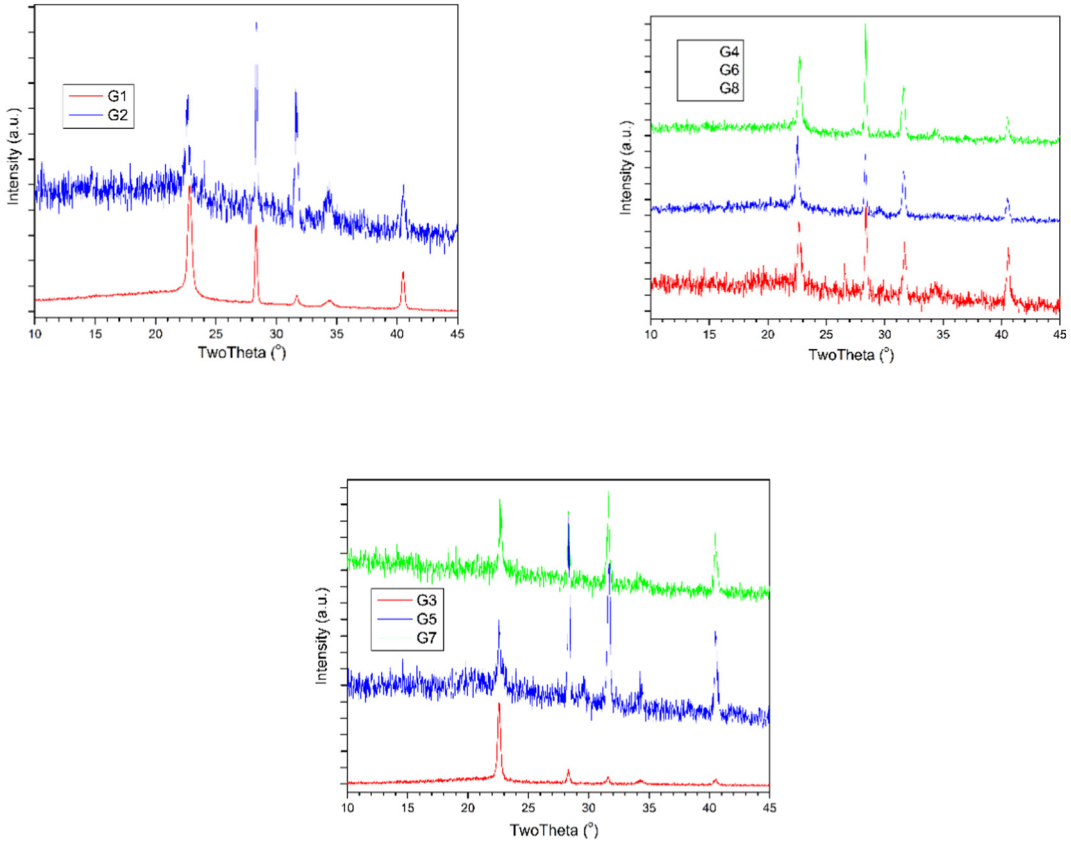


**Fig. 2.** XRD patterns of the raw *R.o.* (G1); mechanically pretreated *R.o.* with zeolite (G2); thermally pretreated *R.o.* without zeolite at 90 °C, 180 min (G3); thermally pretreated *R.o.* with zeolite at 90 °C, 180 min (G4); thermally pretreated *R.o.* without zeolite at 120 °C, 45 min (G5); thermally pretreated *R.o.* with zeolite at 120 °C, 45 min (G6); thermally pretreated *R.o.* without zeolite at 150 °C, 20 min (G7); thermally pretreated *R.o.* with zeolite at 150 °C, 20 min (G8).

amorphization process, which partially could explain literature reports stating that low temperatures (<80 °C) could even decrease the BMP of macroalgae (Barbot et al., 2015; Wormald et al., 1996). From this view, we could expect that during the treatment at 150 °C, a crystallinity loss similar to that achieved at 120 °C occurred followed by a reordering process, explaining the less severe drop in crystallinity observed at 150 °C. On the other hand, zeolite-assisted milling process alone produced a large decrease in crystallinity, from 80 in G1 to 55 in G2, similar to that observed after thermal treatment at 120 °C. However, when the thermal pretreatment was applied to the milled biomass with zeolite the crystallinity of cellulose increased, regardless of the temperature. It is known that algal cellulose is mainly Iα-type and that can be converted to the most stable Iβ-type upon several treatments (Horii et al., 1987). It is then reasonable to speculate that in general, after the large amorphization produced by the zeolite-assisted milling, the thermal treatments rearrange the cellulose chains in the form of Iβ-type. These results suggested that the variable time upon the thermal pretreatments would not be critical in order to obtain better results when these treatments are applied to the biomass milled with zeolite. However, it may need to be taken into consideration if zeolite is not used during milling.

Furthermore, FTIR analysis of all samples was carried out in order to explore the changes in structural features and functional group modifications as a result of the thermal and mechanical pretreatment steps. Fig. 3a and b demonstrated that the pretreated *R.o.* showed the same spectral behaviour with different transmission bands and a slight difference in terms of intensity. The presence of cellulose I was also confirmed with FTIR results, by identifying the usual bands appearing in lignocellulosic biomass (Kassaye et al., 2017; Douissa et al., 2013), as shown in Fig. 3a. All samples presented a strong broad absorption band from at 3000 to 3700 $cm^{-1}$ due to the -OH stretching vibrations in cellulose, hemicellulose and lignin. The transmissions at 2970 and 2850 $cm^{-1}$ were attributed to the existence of the functional group alkane (C—H stretching) in vibrations in cellulose, hemicellulose and lignin. A typical peak associated to cellulosic and hemicellulosic structure was found at 1730 $cm^{-1}$ (stretching vibration of the carbonyl group C═O). Also, the aromatic skeletal vibration of lignin was evidenced in the absorption from 1650 to 1400 $cm^{-1}$. The band at 1626 $cm^{-1}$ corresponded to the bending mode of the absorbed water in the structure of cellulose but it can also be assigned to the absorption of carboxylic groups and the peak at 1432 $cm^{-1}$ to shear vibration of the carboxyl link or $CH_2$ bending. The band at 1272 $cm^{-1}$ corresponds to glucose ring plus C═O stretching. Another dominant band at 1044 $cm^{-1}$ which corresponds to the stretching vibration of the link C-O-C of cellulose was observed in all samples due to that the pretreatments have not significantly affected the cellulose content of the biomass.

### 3.2. Effect of pretreatments on AD performance and methane yield

Fig. 4 shows the accumulated methane production of control and pretreated *R.o.* over time. The methane yield of the control tests (G1), the raw macroalgae, was 180 (34) $NL_{CH4}$ kg $SV^{-1}$, which is in accordance with previously reported studies on the same or similar seaweeds (de la Lama-Calvente et al., 2021; Hessami et al., 2019). At the end of the

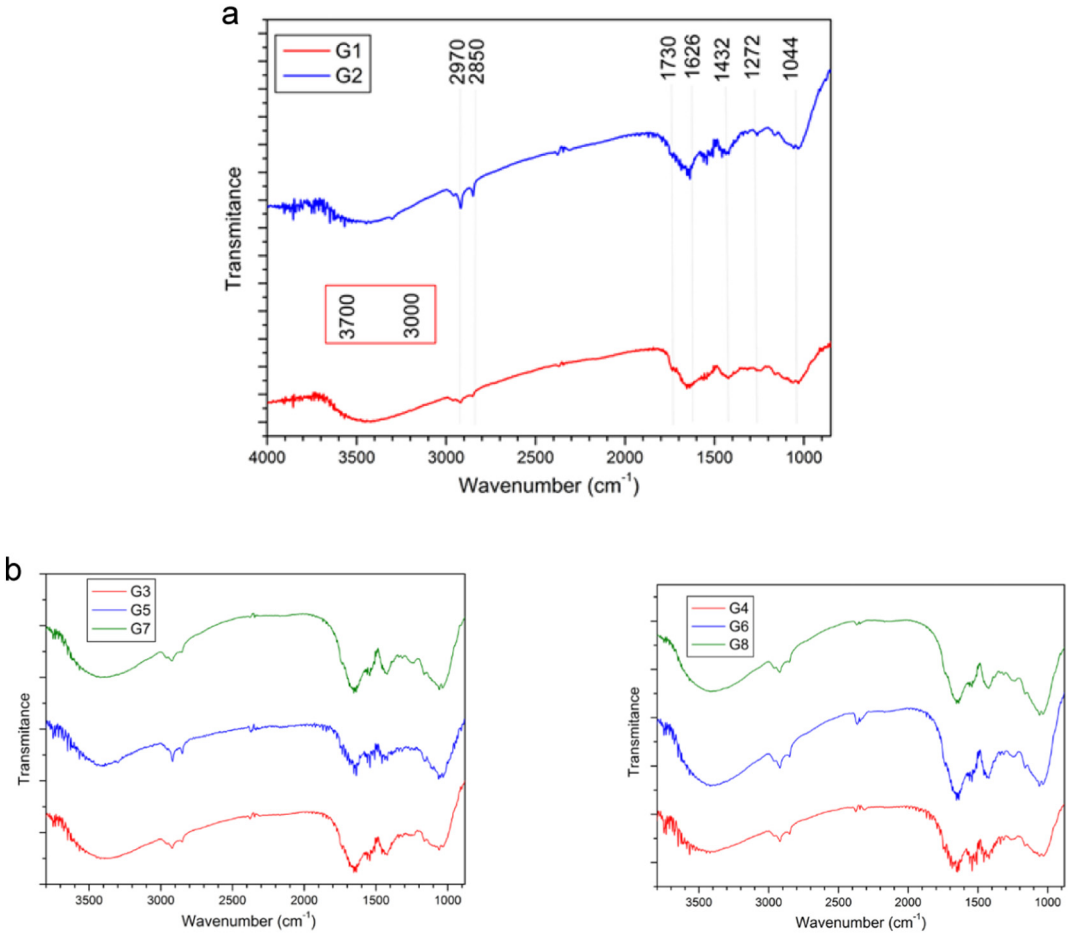


**Fig. 3.** FTIR analysis of control and pretreated *R.o.*
a. FTIR analysis of *R.o.* control (G1) and *R.o.* & zeolite (G2)
b. FTIR analysis of thermally pretreated *R.o.* without zeolite at 90 °C, 180 min (G3); thermally pretreated *R.o.* with zeolite at 90 °C, 180 min (G4); thermally pretreated *R.o.* without zeolite at 120 °C, 45 min (G5); thermally pretreated *R.o.* with zeolite at 120 °C, 45 min (G6); thermally pretreated *R.o.* without zeolite at 150 °C, 20 min (G7); thermally pretreated *R.o.* with zeolite at 150 °C, 20 min (G8).

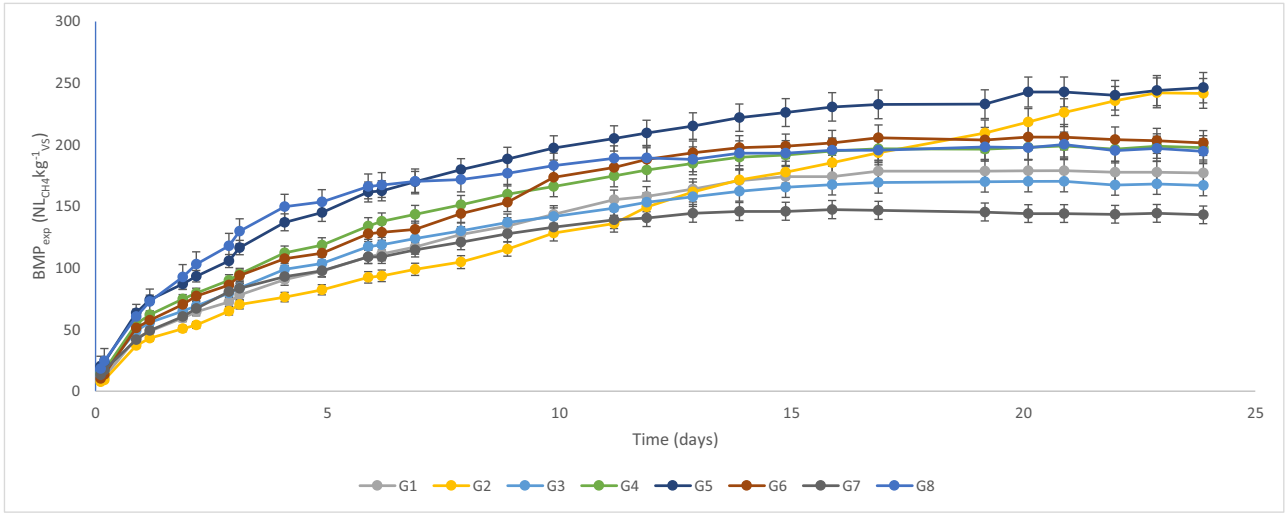


**Fig. 4.** Methane yield versus time curves for *Rugulopteryx okamurae* (*R.o.*) and the different pretreatments tested.
Raw *R.o.* (G1); mechanically pretreated *R.o.* with zeolite (G2); thermally pretreated *R.o.* without zeolite at 90 °C, 180 min (G3); thermally pretreated *R.o.* with zeolite at 90 °C, 180 min (G4); thermally pretreated *R.o.* without zeolite at 120 °C, 45 min (G5); thermally pretreated *R.o.* with zeolite at 120 °C, 45 min (G6); thermally pretreated *R.o.* without zeolite at 150 °C, 20 min (G7); thermally pretreated *R.o.* with zeolite at 150 °C, 20 min (G8).

experiment, the highest methane yields obtained were found for the milled algae with zeolite (G2) and for the thermally pretreated algae at 120 °C during 45 min without zeolite (G5), being the values in the range of 240 (28) – 250 (20) $NL_{CH4}$ kg $SV^{-1}$ and with no significant differences observed between both pretreatments. In both cases, the methane yield, compared with the untreated alga, was improved by 35 %. These two pretreatments showed the highest biodegradability too, representing 49–50 % of the theoretical BMP based on $COD_{total}$ and 44–46 % based on elemental analysis, which meant an improvement of 12–13 % ($COD_{total}$) and 15–17 % (elemental analysis) when compared with the untreated algae (actual results are shown in Table S2).

These results were found to be in accordance with the crystallinity index obtained from the XRD analysis, which showed similar values for both G1 (55) and G5 (54) tests. Moreover, Fig. 5 shows a strong relationship between the crystallinity index of cellulose and the methane yield. This relation fits a linear progression where the lower the crystallinity the higher the methane production, which is in accordance with other studies (Hendriks and Zeeman, 2009). Only one case showed a non-linear progression (G7) with a methane yield much lower than expected (reasons for this are discussed below). Nevertheless, this figure confirmed that the two best performances (G2 and G5) also showed the lowest crystallinity index. Additionally, based on a preliminary economic assessment (Table S5), these two assays are the most profitable methods, although, as it is discussed in the Supplementary Material document, the variation on energy price and zeolite through time may be considered, as slight changes may provoke these treatments to not be as profitable as the control (G1).

### 3.2.1. Effect of thermal pretreatments

As shown in Fig. 4, temperature had an effect on the BMP of *R.o.* The highest methane yield (250 (20) $NL_{CH4}$ kg $SV^{-1}$) was achieved at the medium selected temperature (120 °C, 45 min), showing as well the highest biodegradability (50 %, $COD_{total}$). These values represent a 35 % improvement on methane yield and a 13 % improvement on biodegradability when compared with the control test (G1). Yazdani et al. (2015) reported an increase in methane yield of 22 % when the brown macroalgae *Nizimuddinia zanardini* was pretreated with hot water at 121 °C for 30 min. When

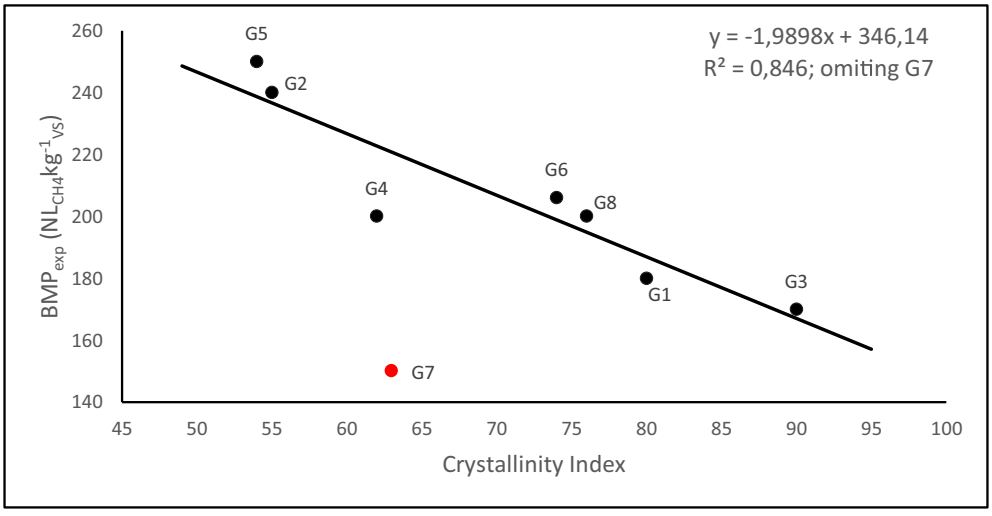


**Fig. 5.** Relationship between crystallinity index and biomethane yield during AD of raw *R.o.* (G1); mechanically pretreated *R.o.* with zeolite (G2); thermally pretreated *R.o.* without zeolite at 90 °C, 180 min (G3); thermally pretreated *R.o.* with zeolite at 90 °C, 180 min (G4); thermally pretreated *R.o.* without zeolite at 120 °C, 45 min (G5); thermally pretreated *R.o.* with zeolite at 120 °C, 45 min (G6); thermally pretreated *R.o.* without zeolite at 150 °C, 20 min (G7); thermally pretreated *R.o.* with zeolite at 150 °C, 20 min (G8).

*Saccharina latissima* was pretreated by steam explosion at medium temperature (130 °C, 10 min) also yielded the highest methane production and improved it against the untreated algae by 20 % (Vivekanand et al., 2012). As it has been discussed earlier, these improvements could be linked to the reduction of cellulose crystallinity rather than cellulose breakdown. Time could potentially be a critical factor that has not been considered in the present study at each selected temperature, however, Thompson et al. (2020) reported that, although, the methane yield from pelagic *Sargassum* increased with the pretreatment time at 120 °C, this increment decreased as the time increase, e.g. from 10 min to 20 min the methane yield improved by 12 % approximately, while from 20 min to 30 min the improvement was <6 %. Then, it is reasonable to assume, that if the pretreatment time is reduced the methane yield would be lower, while if increased, the improvement could not be significant or even not sufficient to compensate the extra energy consumed (preliminary economic assessment is included in the Section 4 of the Supplementary Material document).

When *R.o.* was treated at the lowest temperature (90 °C, 180 min), there was no observed impact on the methane yield (170 (7) $NL_{CH4}$ kg $SV^{-1}$), which is in accordance with a similar study stating that low temperatures (<80 °C) could even decrease the methane production from macroalgae (Barbot et al., 2015). Besides, this treatment provoked an increase of the crystallinity index, confirming that low temperatures do not have an observable effect on the breakdown of cellulose, and the produced methane is derived from the already available material in the biomass, which also support the idea that time would not be a significant variable at 90 °C. However, when higher temperatures were applied to the algal biomass (150 °C, 20 min), a negative effect on methane yield was observed (150 (10) $NL_{CH4}$ kg $SV^{-1}$). Although higher temperatures could potentially increase the breakdown of fiber and, therefore, the soluble matter (Lizasoain et al., 2016), it has been observed that high temperatures have a negative effect on AD due to the formation of refractory and Maillard's reaction compounds and an increase in the concentrations of $NH_3$-N, short-chain fatty acids and phenolic compounds which act as inhibitors for the AD process (Thompson et al., 2020). This could explain the low methane yield of the pretreated algae biomass at the highest selected temperature (G7) despite the observed reduction in the crystallinity index, which seemed to be not enough to overpass the afore-mentioned issues (Fig. 5). Moreover, based on literature reported results (Thompson et al., 2019; Thompson et al., 2020), a decrease in the pretreatment time at 150 °C could potentially slightly enhance the obtained methane yield by reducing the inhibitory compounds production, however, times below 15 min would not guarantee treatment homogenization as it was observed in preliminary experiments.

Finally, the digestates presented pH (7.70–7.89), TA (4590–4800 mg $CaCO_3$ $L^{-1}$) and TAN (760–830 mg $NH_3$-N $kg^{-1}$) values within the optimum range for a stable AD process (Holliger et al., 2016) regardless the biomass was pretreated or not (actual digestate analysis are shown in Table S3), suggesting that thermal pretreatment of the invasive seaweed *R.o.* is a viable option and an adequate process previous to its AD process. Moreover, as it is shown in the Section 3 of the Supplementary Material document, the digestate characterization allows it use as a biofertilizer in all the regions assessed (Table S4).

#### 3.2.2. Effect of zeolite added during milling step

As shown in Fig. 4, the highest methane yield (240 (28) $NL_{CH4}$ kg $SV^{-1}$) was obtained when the macroalgae was milled down with zeolite for 30s but no thermal pretreatment was applied. This could be, as it has been mentioned above, due to the fact that after the large amorphization produced by the zeolite-assisted milling, the use of temperature rearranged the cellulose chains in the form of Iβ-type. This simple and novel method improved the BMP by 35 % and the biodegradability by 12 % ($COD_{total}$), and it could be linked to the cellulose crystallinity reduction (Fig. 5). Rodríguez et al. (2018) also showed an improvement of 74 % in methane production when *Pelvetia canaliculata* was pretreated for 60 min in a modified Hollander beater with no inert material added, although only an improvement of 6 % was reported when the pretreatment lasted 30 min.

On the other hand, no significant differences in methane production (200 (16) – 210 (2) $NL_{CH4}$ kg $SV^{-1}$) were observed when the algal biomass was milled with zeolite and then thermally pretreated, despite the temperature, although, the BMP was improved by 11–15 % when compared against the control test (G1). If compared with the only thermally pretreated assays, the use of zeolite had a positive effect at 90 °C (G4) and 150 °C (G8), showing a methane yield increase of 16.9 % and 35.8 %, respectively. Although, at 90 °C a decrease in the crystallinity index was observed, and, thus, explaining the increase in the methane production, this was not the case at 150 °C. As discussed above, when the thermal pretreatment at higher temperature (G7) was applied, a drastic reduction in methane was observed due to the multiple inhibitors that could be potentially produced. However, when the zeolite was added (G8), these toxic compounds could be adsorbed on the material surface preventing its effect on microorganisms during AD and attaining higher biomethane yields, despite the higher crystallinity index.

Furthermore, when compared with thermal pretreatment at 120 °C (G5), the addition of zeolite (G6) diminished the methane production by 16.3 %. This result suggested that zeolite may act too as a catalyst increasing the production of both soluble organic matter and toxic compounds in the AD process. Zeolites have been widely studied for their catalytic properties in the fields of petroleum refining, fine chemicals, and environmental protection (Wang et al., 2022). Moreover, zeolite catalyst enhanced the aromatic fraction and reduced the phenol and its derivatives during oil refining, as well as being a suitable heterogeneous catalyst during the transesterification of fatty acids (Orege et al., 2022). However, to the best of our knowledge no studies had proved the effect of heterogeneous catalyst on Maillard reactions, the production of refractory compounds or the release of $NH_3$-N and other toxic compounds. Results reported in this study encourage further research in this field in order to provide a better understanding of the phenomenon observed.

Finally, digestate characterization results confirm the stability of the performance (Table S3) and its potential use as biofertilizers in all the regions assessed (Table S4).

### 3.3. Effect of pretreatments on kinetic modelling

#### 3.3.1. First-order kinetic model

Table 2 summarizes the kinetic parameters obtained from Eq. (1) for the AD of the different substrates tested. As can be seen, the low values of the standard deviations, standard errors of estimates, the percentages of errors (lower than 4.5 %), and the high determination coefficient (higher than 0.991) values proved the appropriate fit of the experimental results to the proposed model. The error was defined as the difference in percentage between the experimental and the predicted or theoretical ultimate methane yield.

As can be seen the *k* value increased 28.9 %, 19.8 % and 58.4 % with respect to untreated *R.o.* biomass when the temperature of the thermal pretreatment was augmented to 90 °C, 120 °C and 150 °C, respectively. The highest value (0.26 $d^{-1}$) was achieved for the highest temperature used in the pretreatment (150 °C), while the lowest value was found for the untreated macroalgal biomass (0.16 $d^{-1}$).

A recent study (Ibarlucía et al., 2021) also showed the BMP of two green macroalgae from the South Atlantic Sea such as *Codium* sp. (Codioceae) and *Ulva* sp. (Ulvaceae). The first-order kinetic model was also found adequate for adjustment of the experimental data ($R^2$ > 96 %), the kinetic constant for *Codium* sp. being 124 % higher than for *Ulva* sp. The higher methane production found for *Codium* sp. (205 mL CH4 $g^{-1}$ VS) demonstrated a higher biodegradability of this macroalga, which is attributed to rich in sulphated anionic polysaccharide composition, which decreases the activity of methanogenic microorganisms (Ibarlucía et al., 2021). In the same way, a higher first-order kinetic constant (*k*) value (2.5 $day^{-1}$) was found during the batch anaerobic digestion of the *Laminaria digitate*, a large brown seaweed, which is common in Irish coastal waters. This high value can be attributed to the negligible concentrations of lignin in its structure

**Table 2**
Values of the kinetic constant obtained from the first-order model for the different substrates tested. Values represent the mean (standard deviation).

| Substrate | $G_{max}$ ($NL_{CH4}kg^{-1}$ VS) | k ($d^{-1}$) | $R^2$ | S.E.E. | Error (%) |
|---|---|---|---|---|---|
| *R.o.* control (G1) | 183 (3) | 0.166 (0.008) | 0.993 | 6.837 | 3.7 % |
| *R.o.* (90 °C, 180 min: G3) | 168 (2) | 0.21 (0.01) | 0.991 | 7.174 | 1.1 % |
| *R.o.* & zeolite (90 °C, 180 min: G4) | 198 (2) | 0.205 (0.009) | 0.992 | 7.647 | 0.5 % |
| *R.o.* (120 °C, 45 min: G5) | 239 (3) | 0.19 (0.01) | 0.991 | 10.023 | 2.8 % |
| *R.o.* & zeolite (120 °C, 45 min: G6) | 210 (3) | 0.173 (0.009) | 0.992 | 8.849 | 4.4 % |
| *R.o.* (150 °C, 20 min: G7) | 145 (1) | 0.26 (0.01) | 0.993 | 5.437 | 1.4 % |
| *R.o.* & zeolite (150 °C, 20 min: G8) | 193 (1) | 0.34 (0.01) | 0.996 | 5.454 | 0.5 % |
| *R.o.* & zeolite (G2) | 330 (25) | 0.054 (0.006) | 0.989 | 10.82 | 35.2 % |
| *R.o.* & zeolite (first-step to 6.1 d) | 97 (4) | 0.41 (0.04) | 0.993 | 3.92 | 4.4 % |
| *R.o.* & zeolite (2nd step, from 7.8 d) | 260 (16) | 0.036 (0.002) | 0.998 | 2.69 | 9.1 % |

R.o.: *Rugulopteryx okamurae*; S.E.E.: Standard Error of Estimate; $G_{max}$: Ultimate methane production ($NL_{CH4}kg^{-1}$ VS); *k:* specific rate constant or apparent kinetic constant ($days^{-1}$); $R^2$: Determination coefficient; Error (%): difference (in percentage) between the experimental and calculated ultimate methane production.

and the presence of easily degraded storage polysaccharides (laminarin and mannitol) (O'Shea et al., 2016).

The maximum *k* value (0.34 $d^{-1}$) was found when the *R.o.* biomass was milled with zeolite and pretreated at 150 °C for 20 min (G8). This value was 31 % higher than that achieved when this same pretreatment was applied without zeolite (G7) (0.26 $d^{-1}$). Despite the low accumulated methane production of G8 and G7, due to the reasons widely discussed above, these high constant rates indicated that higher temperatures increased the solubility of biodegradable compounds and that these are consumed in a very early stage, even before any inhibition can take place. Moreover, these results confirmed that zeolite may absorb toxic compounds on its surface.

For the other two temperatures assayed (90 and 120 °C), there were no significant differences in the *k* values when the zeolite was used. Thus, at these temperatures, no significant differences in the soluble easily-biodegradable content are observed regardless of the use of zeolite. However, in the long run, the accumulated methane showed higher differences due to, mainly, a decrease in the cellulose crystallinity (for the experiments treated at 90 °C) and the release of toxic compounds (for the experiments treated at 120 °C), as discussed before.

A special kinetic behaviour was observed when the zeolite was used without any thermal pretreatment (G2), in this case, two different stages in the plot of methane production versus time were observed, a first step from the start of the experiment up to 6.1 days in which the most easily biodegradable organic fraction is degraded and a second stage from day 7 up to the end of the experiment in which the more difficult fraction to biodegrade is converted into methane. The kinetic constants of both stages were found to be 0.41 and 0.036 $days^{-1}$, respectively, when the single first-kinetic model was applied. However, a two-substrate kinetic model seems more appropriate for this behaviour and, thus, it was applied and its discussion is detailed below.

### 3.3.2. Two-substrates kinetic model

As a consequence of the shape of the methane production-time curves for the case of the use of zeolite without thermal pretreatment (G2), the two-substrates kinetic model was also applied. This model allows describing the evident existence of rapid and slowly degradable material. A considerable increase in the kinetic constant value for the rapid biodegradation stage ($k_{rapid}$, 1.8 (0.4) $d^{-1}$) was observed when compared to the value found for the slow biodegradation stage ($k_{slow}$, 0.012 (0.004) $d^{-1}$). The $R^2$ value was higher than 0.999 in this case and the low value of the standard error of the estimate (3.931) also specified a well-suited of experimental data to this proposed model in this experiment.

The increase in the values of the kinetic constant of the faster stage in contrast with the slower stage was also recently observed during the anaerobic co-digestion of different combinations of microalgae and waste activated sludge and the digestion of both substrates individually (Scarcelli et al., 2020). In any case, the values of $k_{rapid}$ and $k_{slow}$ obtained for the seaweed (*R.o.*) when milled with zeolite were always higher than that achieved for the microalgae individually. These results confirmed that zeolite allowed for achieving higher hydrolysis rates due to the synergistic effects of the size reduction and the conversion of the crystalline to the amorph form of cellulose as was discussed before and supported by the earlier study of Oh and Kim (1987).

### 3.3.3. Transference function model

Table 3 shows the TF model parameters obtained for the different substrates tested in this study. The parameters $B_{max}$, $R_{max}$, and $\lambda$ were calculated for each one of the runs studied using the nonlinear regression approach with the software SigmaPlot 11.0. The low values of the standard deviations, standard errors of estimates and the high determination coefficient values demonstrate the appropriate fit of the experimental data of methane production-time to the suggested model. The high accuracy of prediction of the methane production by the proposed model shows that future measurements will fall within the predicted outcome for the *R.o.* biomass (untreated and thermally pretreated).

The obtained lag times ($\lambda$) were found to be almost zero in all the cases studied, indicating a fast consumption of the easy and most available biodegradable components of the different substrates assayed in all the AD processes studied.

An increase of 31.2 % in the maximum methane production rate, $R_{max}$, was observed when the temperature in the pretreatment augmented from 90 °C to 120 °C (without zeolite addition; G3 and G5, respectively), achieving a value of 47 (2) $NL_{CH4}\ kg^{-1}\ SV\ d^{-1}$ at the last temperature. However, when the temperature increased to 150 °C (G7), a significant decrease in

**Table 3**
Values of the parameters obtained from the Transference Function (TF) model for the different substrates studied. Values represent the mean (standard deviation).

| Substrate | $B_{max}$ ($NL_{CH4}kg^{-1}$ VS) | $R_{max}$ ($NL_{CH4}kg^{-1}$ VS $d^{-1}$) | $\lambda$ (d) | $R^2$ | S.E.E. | Error (%) |
|---|---|---|---|---|---|---|
| *R.o.* control (G1) | 183 (3) | 30 (1) | $3.6{\cdot}10^{-9}$ | 0.993 | 6.962 | 3.7 % |
| *R.o.* (90 °C, 180 min: G3) | 168 (2) | 36 (1) | $3.8{\cdot}10^{-9}$ | 0.991 | 7.303 | 0.5 % |
| *R.o.* & zeolite (90 °C, 180 min: G4) | 198 (2) | 40 (1) | $3.5{\cdot}10^{-9}$ | 0.993 | 7.788 | 0.6 % |
| R.o. (120 °C, 45 min: G5) | 239 (4) | 47 (2) | $4.2{\cdot}10^{-9}$ | 0.991 | 10.207 | 2.8 % |
| *R.o.* & zeolite (120 °C, 45 min: G6) | 210 (4) | 36 (2) | $3.5{\cdot}10^{-9}$ | 0.992 | 9.011 | 4.6 % |
| *R.o.* (150 °C, 20 min: G7) | 145 (1) | 38 (1) | $2.7{\cdot}10^{-9}$ | 0.993 | 5.537 | 1.4 % |
| *R.o.* & zeolite (150 °C, 20 min: G8) | 193 (1) | 66 (2) | $1.5{\cdot}10^{-9}$ | 0.996 | 5.554 | 0.5 % |
| *R.o.* + zeolite (G2) | 330 (30) | 17 (1) | $1.0{\cdot}10^{-8}$ | 0.989 | 11.026 | 35.2 % |

*R.o.: Rugulopteryx okamurae; $B_{max}$:* is the ultimate methane production; $R_{max}$: is the maximum methane production rate; $\lambda$ is the lag time. S.E.E.: Standard Error of Estimate; $R^2$: Determination coefficient; Error (%): difference (in percentage) between the experimental and calculated ultimate methane production.

the $R_{max}$ value to 38 (1) $NL_{CH4}\ kg^{-1}\ SV\ d^{-1}$ was found. The same trend was observed for the ultimate methane production, $B_{max}$, confirming the discussed effect of zeolite and temperature over the biomass.

On the other hand, a lower maximum methane production rate, $R_{max}$, value (12 $NL_{CH4}\ kg^{-1}\ SV\ d^{-1}$) was achieved in the AD of the perennial grass *Miscanthus floridulus* after a hot-water (low temperature) pretreatment performed at 95 °C for 10 h, as the impact of this hot water pretreatment on the hemicellulose (5.04 %) and cellulose (1.23 %) reductions was very low (Fu et al., 2018).

The maximum $R_{max}$ value was achieved after thermal treatment of 150 °C for 20 min and zeolite addition (G8) (66 (2) $NL_{CH4}\ kg^{-1}\ SV\ d^{-1}$), a value 75 % higher than that achieved at this same temperature but without zeolite (G7). This highest value of $R_{max}$ at 150 °C when zeolite is added during the milling process could be related to the adsorption of toxic compounds on the zeolite surface as previously discussed.

## 4. Conclusions

This study showed that the anaerobic digestion of newly developed mechanically pretreated and thermally pretreated invasive alien macroalgae, such as *R. okamurae*, is a viable process embedded in a circular economy system, able to convert the more frequent environmental problem of a massive invasion of alien macroalgae into renewable energy and biofertilizers.

Moreover, the crystallinity index of cellulose analysis shows a linear relationship with the methane yield. With only one exception (G7), which could be explained considering the higher presence of inhibitory compounds released/produced due to the high severity of the pretreatment. This method could be adopted as a preliminary analysis able to identify better pretreatment conditions for those biomasses where cellulose takes a main role regarding the produced methane.

Furthermore, zeolite can be used in AD systems not only due to the well-known benefits (i.e. nutrient removal, microbial immobilizer or biogas purifier), but as an abrasive during the milling process of biomass. This would increase the amorphization of cellulose and the subsequent methane yield. However, a further thermal treatment decreases the methane yield due to the recrystallization of cellulose, regardless the temperature.

Additionally, the selected temperature during pretreatments affects greatly both the crystallinity of cellulose and the release of inhibitory compounds for the AD process. At 120 °C an improvement can be seen and it could be related mostly to cellulose amorphization, however, 90 °C pretreatment foments cellulose recrystallization while at 150 °C inhibitory compounds are produced, and thus the observed negative effect on methane yield. Although different thermal pretreatment times for each temperature have not been tested, it is expected that different times will have worse results or at best some slight improvement, which could not be enough to compensate for the extra energy needed.

The results obtained encourage further research on how the zeolite behaves during the milling process and the thermal treatment, separately and in combination.

Finally, the experimental accumulated methane production of the *R.o.* biomass (control and thermally treated with and without zeolite) was well described by the first-order and Transference Function (TF) models. However, a two-substrate kinetic model was needed to be applied to the AD of the biomass milled with the zeolite-alone test, highlighting the effect of zeolite increasing the content of the easily-degradable compound and reducing the cellulose crystallinity.

## CRediT authorship contribution statement

**David De la Lama-Calvente:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Visualization, Writing – original draft. **María José Fernández-Rodríguez:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing – original draft. **Menta Ballesteros:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft. **Ángel Rabdel Ruiz-Salvador:** Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – original draft. **Francisco Raposo:** Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing – original draft. **José Carlos García-Gómez:** Conceptualization, Data curation, Formal analysis, Investigation, Supervision, Validation, Writing – original draft. **Rafael Borja:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – original draft.

## Data availability

Data will be made available on request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.158914.

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