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**MASTER THESIS**

**FOOD PROCESSING WASTEWATER TREATMENT USING  
BLACK SOLDIER FLY LARVAE**

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## **ABSTRACT**

The use of Black Soldier Fly (BSF) larvae has been recently studied as a promising biological treatment process for high organic content wastewater. Being wastewater quality difficult to control, as it may vary dramatically in time and from wastewater generating process to another, artificial wastewater has been mainly used to better comprehend the treatment process. In this study process performance was investigated in the treatment of real wastewaters from different food-processing industries, such as Bakery, Brewery, Dairy, Juice production, Slaughterhouse and Winery.

The compatibility of BSF larval process with different food-processing wastewaters was evaluated by measuring both the removal rates of organic substance (mg/day/larva), eventually providing design parameters for real scale treatment plants, and larval growth in terms of wet weight and percentage of prepupation. The feeding substrates were monitored for total organic carbon (TOC), chemical oxygen demand (COD), biochemical oxygen demand (BOD<sub>5</sub>), total Kjeldahl nitrogen (TKN), ammonia and phosphate.

## **1. THESIS BACKGROUND**

Solid waste management and its stabilization are a challenging task as it requires improved collection and treatment strategies. From the past decades, there is a huge emphasis on valorisation of waste leading to a sustainable approach for solid waste management. Use of BSF (*Hermetia illucens*) larvae in organic waste composting is an environment friendly approach which holds enormous potential and therefore, is an interesting emerging waste treatment technology. The BSF larvae composting promotes both resource recovery and value-added products generation, developing new economical dimensions for the industrial sector. The biological treatment process based on the metabolism BSF larvae proved to be a favourable technique for the treatment of high organic content wastewater too.

The challenge is dealing with large quantities of nutrients, organic carbon, suspended and dissolved solids, organic content values ranging in the thousands of milligrams per liter, and some like cheese production, winery and olive milling can be in the tens to hundred thousand for COD. In conventional processes, significant volumes are treated and huge production of waste, in form sludges and digestate, are produces. In addition, seasonality of production is a variable to consider too. The BSFL process represents a solution to be used to treat high organic contents loads, but also to benefit from in terms of resource recovery.

In this study, food processing wastewater feedings are considered in order to evaluate the compatibility of larvae with tested feedings and possibly understand how the feeding can be optimized by mixing appropriately the wastewaters to get a more balanced nutrition. There are many factors that affect the growth BSF larvae. Assuming unlimited access to a chosen material, development of insect larvae depends in the first instance on the presence of essential nutrients. Shortages, lack or abundance of essential nutrients might result in reduced growth and lowered survival rates (C. Lalander et al, 2018). Therefore, it's crucial to identify the substrate parameters, in particular in terms of nutrients, that influence the fly larvae composting process.

### **1.1 Life cycle of BSF**

The Black Soldier Fly has become one of the most important insects in the world for bioconversion (De Smet et al., 2018). The BSF can today be found in many countries with year-round warm, tropical or sub-tropical climate (Gold et al., 2018).

The potential of BSFL to process organic waste has gained particular attention. In fact, the BSF larvae are more voracious eaters of decaying organic matter than the other fly species and this depends on their morphological and biological characteristics. *H. illucens* has five distinct

developmental stages: egg, larva, pupa, prepupa and adult (Figure 1). In nature, it lays its eggs in dry areas near wet and rotting organisms. The incubation time of the eggs is approximately 4 days and varies with season, region, and temperature (Liu et al, 2019).

*H. illucens* begins feeding immediately upon hatching, with consumption rates importantly increasing after the 3rd instar. Contrary to what happens to the *Musca domestica* Linnaeus, when the BSF larvae reach the 6th instar, they cease feeding (Liu et al., 2019). The larval stage is the only feeding stage: the adult fly does not feed and survives only on its body fat reserve, limiting the risk of disease transmission. Under optimal feeding conditions, the larval stage lasts 13 to 18 days. However, it might take up to 4 months in case of rough conditions. According to De Smet et al., larvae have six instars<sup>1</sup>, and the larvae range in size from 1.8 mm to 20 mm.

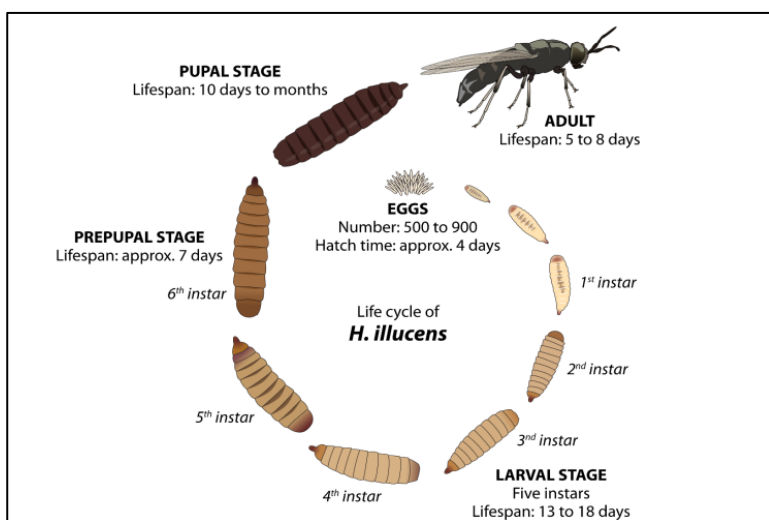


Figure 1 The life cycle of *H. illucens*. (De Smet, et al., 2018)

Once they become prepupae, the colour of larvae starts to darken and they self-harvest by leaving the wet feeding for a drier prepupation site (Grossule and Lavagnolo, 2019). Subsequently they become adults. The female BSF looks for a dark place close to organic matter in decomposition to lay between 500 and 900 eggs, in order to let the larvae emerging from the eggs have sufficient food provision nearby. The performance and nutrition of the *H. illucens* vary with substrate, and substrates rich in protein and oil are more conducive to the accumulation of protein and lipids in this insect. (Liu et al., 2019).

## 1.2 Effects of food composition on larval growth

The natural diet of BSF larvae include decaying plant and animal material such as decaying fruits, vegetables, rotting food products, heaps of cow, poultry manure, human and animal waste (Singh and Kumari, 2019). In fact, most of the literature investigating on substrate macronutrients

<sup>1</sup> Larval development stages

influence on larval growth focuses on solid waste. Gold et al., 2018 try to understand the influence of macronutrients on process performance (larval development time, weight, and composition) and the influence and fate of microbes and chemicals.

Crude protein and crude fat are the main compositions of BSF prepupa, accounting for about 45% and 30% in dry mass, respectively. The rest smaller part is mineral and carbohydrate. Thus, whether BSFL can accumulate enough protein and fat is critical for its well beings.

Protein in diets is the critical substance controlling BSF larvae transformation into the pupa stage. Gold et al, 2020 state that larvae grown on biowaste higher in proteins have a higher larval weight, bioconversion rate, feed conversion rate and larval protein content, and a lower developmental time and lipid content. BSF larvae grown on biowaste lower in proteins have a higher developmental time to assimilate enough protein and they are smaller. However, too much protein content like in liver and fish may cause malnutrition, leading to side-effects such as high mortality rate. This may be due to high ammonia, urea acid, and H<sub>2</sub>S concentration, which is toxic to BSF larvae, produced by excessive protein in diets used as a main energy supplier.

The other significant component of BSF larvae are lipids, which contain much more energy density but aren't as digestible as protein. Lipids are needed as an energy storage for the adult stage, when they lose their mouth apparatus system. However, larvae don't necessarily acquire fat from diets containing much fat. In fact, when they ingest non- fiber carbohydrates, these undergo hydrolysis which produces simple sugars and organic acids. Therefore, wastewater carbohydrate content influences the BSF larvae lipid content. That's why we could observe relatively high-fat content in BSF larvae reared on organic waste low in fat but high in non-fiber carbohydrates content. In fact, too much fat in a diet may cause a negative or even detrimental effect on BSF larvae.

Carbohydrates have a crucial importance in a rearing diet, since they are assimilated by larvae as organic acids. On low-protein and high-carbohydrate diets, carbohydrates are converted by larvae into lipids and stored in the fat body. BSF larvae produced on comparatively low-protein and high-carbohydrate diets are typically higher in lipids than BSF larvae produced on diets more balanced in carbohydrates and proteins. An explanation of this phenomenon is that BSFL eats more to get enough protein before entering the pupa stage, thus at the same time, consuming a large amount of carbohydrates and resulting heavier weight.

Few studies explored the optimal protein:lipids:carbohydrates ratio favorable for larvae growth. Gold et al, 2020 assess the performance of BSF larvae treatment as applied to six types of waste (mill by-products, human faeces, poultry slaughterhouse waste, cow manure, and canteen and vegetable canteen waste) following the determination of their respective nutritional composition. The formulations contained between 14 and 19% DM of protein and between 13 and 15% DM of

non-fibre carbohydrate (NFC), while nutrient composition of each kind of waste varied significantly (Table 1).

Mean performance of BSFL treatment on the different biowastes are reported in Table 2. All individual biowastes supported the development of BSF larvae and the mean survival rates were 90–99%, while treatment performance varied widely. Waste reduction and protein conversion efficiency were lower for all the wastes in comparison to the poultry feed, and cow manure had the worst performance in all of the performance parameters.

Using vegetable canteen waste and mill by-products resulted in the highest waste reduction even though values were still 17 to 20% lower than for the poultry feed. This could be due to the high NFC content of these two wastes, which are easily digested and absorbed into fly larvae. An interesting finding was that comparison of the waste reduction and larval weight results of mill by-products and human faeces demonstrate that this higher waste reduction did not necessarily result in higher larval weight.

Human faeces got a bioconversion rate that was comparable to the poultry feed, despite having a much lower waste reduction. This was due to an 85% higher waste conversion efficiency than with the poultry feed. BSFL showed a lower performance using poultry slaughterhouse waste than human faeces. The lowest performance was found for cow manure, which was low in protein, NFC, and lipids. In contrast, the human faeces and poultry slaughterhouse wastes were high in protein and lipids. We can affirm that NFC is less important for larval development than high overall nutrient content.

*Table 1 Mean nutrient composition of individual biowastes as percent of dry mass, moisture content in percent, and caloric content as kcal per 100 g dry bio (Gold et al, 2020)*

Wastes	pH	Moisture content	Protein	Non-fibre carbohydrates			Fibre			Lipids	Organic matter	P:NFC <sup>a</sup> ratio
				Total	Glucose	Starch	Total	Cellulose & lignin	Hemicellulose			
Mill by-products	6.2 (0.1)	70.0	14.5 <sup>d</sup> (0.3)	23.2 <sup>b</sup> (0.2)	1.7 <sup>b</sup> (0.0)	21.2 <sup>b</sup> (0.6)	51.7 (0.9)	22.1 (1.0)	29.6 (1.9)	3.0	93.8 <sup>c</sup> (1.3)	1:2
Canteen waste	4.3 (0.0)	74.0 (1.2)	32.2 <sup>b</sup> (0.8)	7.5 <sup>d</sup> (0.7)	3.5 <sup>a</sup> (0.4)	4.0 <sup>d</sup> (0.4)	36.2 (1.4)	22.8 (0.6)	13.4 (0.9)	34.9	93.0 <sup>c</sup> (0.7)	4:1
Human faeces (1)	6.0 (0.0)	76.7 (0.9)	20.1 <sup>c</sup> (0.9)	1.7 <sup>a</sup> (0.1)	1.0 <sup>bc</sup> (0.0)	0.7 <sup>a</sup> (0.1)	27.9 (0.6)	19.5 (0.3)	8.4 (0.6)	20.9	86.4 <sup>b</sup> (0.3)	12:1
Poultry slaughterhouse waste	5.7 (0.1)	66.7 (1.2)	37.3 <sup>a</sup> (0.5)	0.3 <sup>a</sup> (0.1)	0.2 <sup>d</sup> (0.1)	0.1 <sup>a</sup> (0.0)	20.8 (1.9)	9.3 (0.9)	11.5 (2.7)	42.9	94.0 <sup>c</sup> (1.3)	152:1
Cow manure	7.2 (0.1)	87.0 (0.2)	11.1 <sup>e</sup> (0.4)	1.8 <sup>a</sup> (0.6)	0.7 <sup>cd</sup> (0.3)	1.0 <sup>a</sup> (0.4)	58.4 (0.4)	40.9 (1.7)	17.4 (1.2)	4.4	80.7 <sup>a</sup> (0.5)	7:1
Vegetable canteen waste	3.8 (0.0)	82.7 (0.1)	12.1 <sup>e</sup> (0.1)	15.5 <sup>a</sup> (0.9)	3.7 <sup>a</sup> (0.3)	11.6 <sup>a</sup> (0.6)	31.5 (1.8)	24.0 (1.5)	7.5 (0.3)	28.9	92.4 <sup>c</sup> (0.5)	1:1
<b>Mean</b>	<b>5.5 (1.3)</b>	<b>74.5 (10.0)</b>	<b>21.2 (10.2)</b>	<b>8.3 (9.2)</b>	<b>1.8 (1.5)</b>	<b>6.4 (8.4)</b>	<b>37.7 (14.5)</b>	<b>23.1 (10.2)</b>	<b>14.6 (8.2)</b>	<b>22.5 (16.3)</b>	<b>90.1 (5.4)</b>	<b>29:1</b>
Poultry feed (benchmark)	5.7 (0.0)	60.0	19.1 <sup>f</sup> (0.7)	28.5 <sup>a</sup> (0.8)	0.5 <sup>cd</sup> (0.2)	27.5 <sup>a</sup> (1.4)	22.0 (1.0)	8.6 (0.0)	13.5 (1.1)	4.8	98.2 <sup>ab</sup> (4.0)	1:2

In parenthesis: standard deviation for samples where n ≥ 3 and differences between analyses where n = 2.  
<sup>a</sup> results with no shared letter are significantly different from each other.  
<sup>a</sup> P:NFC = ratio of protein to non-fibre carbohydrates (NFC).  
<sup>a</sup> gross caloric content of protein, NFC, and lipids.

Table 2 Mean performance of BSFL treatment on the different biowastes (Gold et al, 2020)

	Survival rate %	Larval weight mg DM	Waste reduction % DM	Bioconversion rate % DM
<b>Individual wastes</b>				
Mill by-products	96.2 (1.5)	41.7 (0.9)	56.4 (1.2)	14.9 (0.3)
Canteen waste	92.3 (3.1)	44.2 (5.9)	37.9 (3.8)	15.3 (2.1)
Human faeces (1)	99.1 (0.6)	58.8 (1.7)	39.1 (1.5)	22.7 (0.6)
Human faeces (2)	96.2 (2.5)	50.2 (1.2)	48.6 (0.3)	18.8 (0.8)
Poultry slaughterhouse waste	90.7 (2.9)	39.4 (0.7)	30.7 (4.7)	13.4 (0.5)
Cow manure	89.8 (7.5)	14.3 (0.4)	12.7 (0.9)	3.8 (0.2)
Vegetable canteen waste	97.5 (2.7)	59.1 (2.6)	58.4 (1.4)	22.7 (1.1)
Poultry feed (benchmark)	97.9 (2.1)	55.6 (5.1)	67.7 (6.9)	21.0 (2.4)

From Table 3, we tried to understand how these relations were changing for increasing larval weight (mg DM) and we can notice that in human faeces the proteins are 12 times carbohydrates, even if larvae had one of the best larval growth performances. We discovered that it makes more sense to consider a protein: (lipids+ carbohydrates) ratio, since carbohydrates undergo hydrolysis process and are assimilated by larvae as organic acids. For human faeces the ratio is 1:1.1. In cow manure, too many proteins are present with respect to carbohydrates+ lipids and this led larvae to develop less than the others. On the other side, too low amount of proteins force larvae to feed more and assimilate more lipids and carbohydrates, increasing their weight. However, if proteins are too low, we can notice a general starvation. To conclude, larvae develop and have a good treatment performance if protein amount is equal or lower to the sum of carbohydrates and lipids.

Table 3 Normalization of nutrients content

Wastes	Normalisation of nutrients contents	
	P	C+L
Cow manure	1.8	1.0
Poultry slaughterhouse	1	1.1
Mill by- products	1	2.2
Canteen waste	1	1.3
Poultry feed	1	2.3
Human faeces	1	1.1
Vegetable canteen waste	1	3.4

The best biowaste thus depends on the objective of the BSFL treatment. Mill by-products and vegetable canteen waste performed best with respect to waste treatment while human faeces and vegetable canteen waste were more indicated with respect to larval biomass production efficiency. The most protein per unit of biowaste was produced using the mill by-products and the canteen

waste. Thus, for facilities targeting insect protein meal production, these wastes would be favourable. Poultry slaughterhouse waste and cow manure resulted in generally scarce performance.

In conclusion, a particular attention should be paid to fibres. Because fibres are indigestible, large amounts of fibres can reduce the process performance by reducing the overall nutrient density for BSF larvae development. This could be one contribution to the lower dry weight waste reduction and longer development time of biowaste high in fibres, such as animal manures. Therefore, biowaste characterisation should consider the function of different carbohydrates and pre-treatment of biowaste before BSF larvae treatment or inoculation at the beginning of BSF larvae treatment with microbes that can degrade fibres, can be an important future research possibility to improve the process performance (Gold et al., 2018).

### **1.3 Resource recovery from larvae**

Due to the increase of population and the economic development of low-, and middle-income countries, waste production is inevitably experiencing a sharp increase. The increase in waste generation is among major concerns in many metropolitan areas around the world. If the waste is not managed well, it can pose serious threats to the environment and can cause issues in public hygiene and health. Foul odour, emission of greenhouse gasses like methane gas, contamination of water due to underground leaching and diseases spread by vectors are some of the consequences from the organic waste mismanagement.

On the other hand, energy demand along with the search for alternative green energy is rising to reduce the dependency on fossil fuels which are non-renewable and are declining in availability. Therefore, it would be an ideal solution to address both problems by transforming organic waste into higher value product. It is in this context that the Black Soldier Fly (BSF) larvae has been considered for the treatment of organic waste. In particular, the larval biomass generated through bioconversion of organic wastes is a high-quality source of fat/oil as well as proteins and is increasingly applied in multiple industries for animal feeding, biodiesel production, soil composting and biopolymers (chitin).

### 1.3.1 Animal feeding

FAO<sup>2</sup> states that about 1000 million t of animal feed is produced globally every year, including 600 million t of compound feed and feed for poultry is the greatest tonnage, followed by pig and cattle feeds.

The worldwide growing consumption of proteins for human and animal nutrition has drawn a considerable amount of attention to insect rearing; sector stakeholders focus on insect potential to convert organic material of low quality into high quality food and feed. FAO stated that the practice of rearing insects on human wastes and using them as a protein source for domestic animals is more environmentally friendly than using the land, water, and energy resources that could otherwise be employed to produce food for humans. BSF larvae or pupae can be incorporated into the diets of poultry, fish, pets, and pigs, serving as prospective alternatives of common feed ingredients, namely soybean and fish-based meal (S.A. Siddiqui et al., 2022).

The importance of fish meal and oil in aquaculture is crucial, but competition with demands for fish for human consumption and depleted fisheries, among other factors, have brought the supplies of fish meal and oil down and costs up, leading fisheries to search for alternatives. BSF larvae's ability to efficiently produce protein-rich edible biomass from potentially protein-poor organic wastes has led many authors to conclude that BSFL can contribute meaningfully to sustainable aquaculture (Wang and Shelomi, 2017).

BSF larvae render nutrient benefits more efficiently than fish meal, therefore can replace the fish meal for animal feeding (A. Singh and K. Kumari, 2019). Singh and Kumari report several studies which replaced traditional fish meal of marine fishes with a certain % of BSF larvae, concluding that no difference is seen in the growth patterns of fishes compared to traditional meals. Moreover, they state that BSF larvae are efficient alternative source replacing fish meal, oil and conventional fat and protein sources to feed poultry animals and fishes.

BSF larvae have also been used in poultry feed as a partial replacement for maize or soy-based feeds, mainly because the species naturally colonizes and breaks down poultry manure and populations are often kept by poultry farms for the purpose of waste management and pollution reduction.

*Kopernik*, a non-profit organization headquartered in Indonesia that finds what works to reduce poverty in the last mile, tested the viability of BSF larvae as a feed alternative for duck breeders in Bali (Fig. 2). They tested four feed compositions and they found that BSF larvae were a

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<sup>2</sup> Food and Agriculture Organization of the United Nations

nutritionally comparable alternative to the current duck feed (concentrate). The analysis results showed that BSF larvae had higher nutritional values compared to the current duck feed. BSF larvae had 61% protein, 27% fat, and 31% crude fiber content while the common concentrate had 37-38% protein, 2-3.5% fat and 6% crude fiber content. They concluded that BSF larvae are a healthy and nutritionally comparable feed alternative to the current duck feed used in Bali.

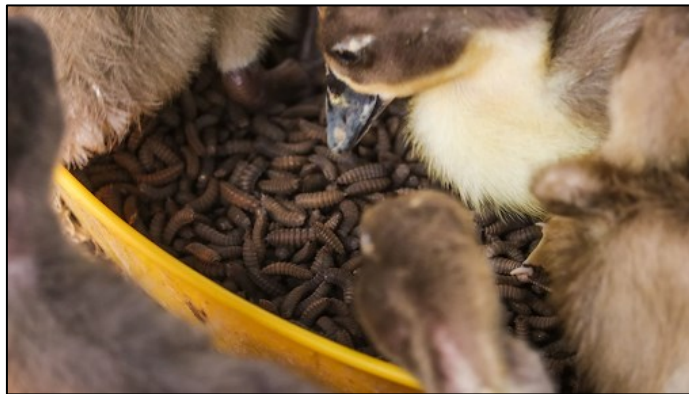


Figure 2 Ducks fed on BSF larvae. (“Black Soldier Fly Larvae-Based Animal Feed | Solutions Catalog Kopernik”)

### 1.3.2 Biodiesel production

Majority of the BSF larvae biomass consists at least 30% of lipid (Leong et al., 2015). Therefore, the bioconversion by BSF larvae is a win-win process not only assisting in waste management but the amount of lipid obtained is upcycled into biodiesel as an alternative feedstock for renewable energy.

Moreover, continuous and large-scale production of biodiesel from food grade oil may cause global imbalance to the food supply and demand market. Thus, biodiesel production using BSF larvae as a feedstock has received a great attention from scientist worldwide.

Biodiesel, a mono alkyl ester (methyl or ethyl ester) of long chain fatty acids derived from renewable lipid such as vegetable oils and animal fats, can be used as a substitution fuel for traditional diesel in any compression ignition engines with little or no modification (Zheng et al., 2011). The fuel is produced by transesterification, that converts fats and oils into biodiesel and glycerin (a coproduct) (Fig. 3). Transesterification process involves a reaction between triglyceride molecules and an alcohol in the presence of catalyst to form esters and glycerol (Ishak and Kamari, 2018).

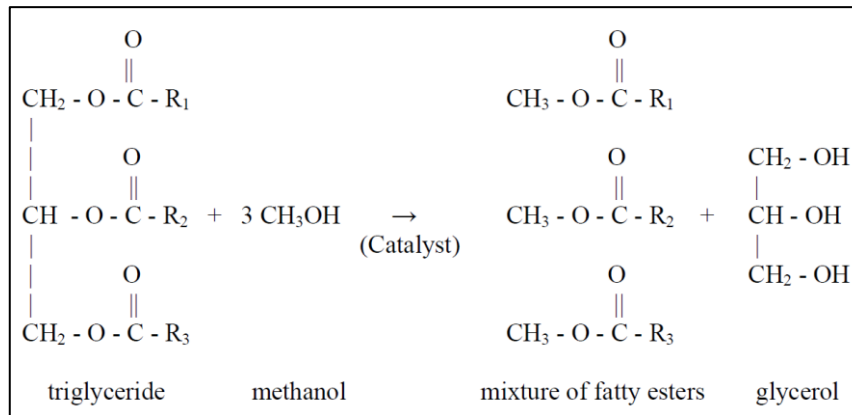


Figure 3 Reaction equation of the transesterification process (“EVC1: Transesterification to Biodiesel”)

Since there are not researches about biodiesel production from BSF larvae fed by food processing wastewater yet, biodiesel from BSF larvae grown on solid food waste will be considered.

Ishak and Kamari (2018) considered BSF larvae cultivated for 20 days on kitchen waste. Cultivation of larvae is performed at a temperature of 25–32 °C and humidity of 60–75%. After 20 days, BSF larvae are separated from the feeding media, inactivated at 105 °C for 5 min and dried in an oven at 60 °C for 24 h. Extractions of BSF larval lipid are performed using petroleum ether<sup>3</sup> as a solvent for 6 h, followed by distillation process to remove remaining solvent. The round bottom flask containing BSF larval lipid is placed in an oven at 103 °C and heated to a constant weight, followed by cooling in a desiccator for 30 min. Then, the weight and percentage of BSF larval lipid produced are determined. The BSF larval lipid is heated on a hot plate at 30 °C, the concentrated phosphoric acid is poured into larval lipid and left for 15 min and deionised water is then added into the mixture. These procedures are important in order to remove impurities. Finally, the BSF larval lipid is centrifuged to separate impurities. High purity of BSF larval lipid is collected. The larval lipid yield extracted from BSF larvae is 56% and sent for transesterification process.

To achieve maximal biodiesel yields, usually, a two-step approach with acid-catalysed esterification and sequential alkaline catalysed transesterification is used for biodiesel production to ensure an efficient conversion of free fatty acids (FFA) into biodiesel, due to high free fatty acid content in the larval lipid (Zheng et al., 2011).

The acid-catalysed esterification step was performed at the following parameters, namely:

1. methanol: oil molar ratio of 10:1,
2. 1% (w/w) sulphuric acid as a catalyst,

<sup>3</sup> Petroleum ether is a light, volatile, petroleum fraction predominantly composed of aliphatic hydrocarbons. Available in various quantities and reagent grades, it is a common liquid hydrocarbon solvent that does not actually contain ether functional groups. (“Petroleum Ether”)

3. reaction time of 41 min and
4. reaction temperature of 50 °C.

The resulted solution is centrifuged at 400 rotation per min for 10 min, whereby the upper layer is then transferred to a conical flask and dried in an oven at 105 °C for 10 min.

The second step, the alkaline-catalysed transesterification, is conducted at optimum experimental parameters, namely:

1. methanol: oil molar ratio of 10:1,
2. 1.1% (w/w) sodium hydroxide as a catalyst,
3. reaction time of 61 min and
4. reaction temperature of 62 °C.

The solution was centrifuged, transferred and dried. The biodiesel yield obtained from transesterification of BSF larval lipid was 97% indicating the feasibility of BSF larvae fed by restaurant kitchen waste to be converted to biodiesel.

The properties of this larval based biodiesel are then determined according to European biodiesel standards and the American Society for Test and Materials (Table 4).

Most of the BSF larval biodiesel properties meet the American and European standards, particularly water content, flash point, kinematic viscosity, density at 15 °C and cloud point. The oxidation<sup>4</sup> stability value of BSF biodiesel (5 h) meets the American Society for Test and Material D6751 standard (>3 h); however, the value was slightly lower than European Standard 14214 (>6 h). The BSF biodiesel has a cetane number of 49, which is within the acceptable range value set by American Society for Test and Materials D6751 ( $\geq 47$ ). In contrast, this value was rather close to the cetane number recommended by European Standard 14214 (>51).

*Table 4 Properties of BSF larval biodiesel*

Properties	Method	Value	ASTM D6751	EN 14214
Oxidation stability (h)	EN 14112	5	>3	>6
Water content (mg/kg)	ASTM D2709	387	<500	<500
Flash point (°C)	ASTM D93	143	>130	>120
Kinematic viscosity at 40 °C (mm <sup>2</sup> /s)	ASTM D445	4.6	1.9-6.0	3.5-5.0
Cloud point (°C)	ASTM D2500	5.6	-3 to 12	-
Cetane number	EN ISO 5165	49	$\geq 47$	>51
Density at 15 °C (kg/m <sup>3</sup> )	EN ISO 3675	875	-	860-900

<sup>4</sup> "Fuel stability" is the resistance of a fuel to degradation processes that can change fuel properties and form undesirable species. A fuel is considered unstable when it readily undergoes these changes.

"Oxidation stability" refers to the tendency of fuels to react with oxygen at temperatures nearer ambient and describes the relative susceptibility of the fuel to degradation by oxidation. (Pullen and Saeed,2012)

It was found that seven major fatty acid methyl esters (FAME) compose the BSF larval biodiesel:

- 85.89% are saturated (lauric acid, myristic acid, palmitic acid, capric acid and stearic acid) FAME;
- 14.10% are unsaturated (oleic acid and palmitoleic acid) FAME.

It is known that oxidation stability of biodiesel products is greatly influenced by fatty acid methyl esters composition, of which saturated fatty acid methyl esters will increase the oxidation stability (Zhang 2012). Furthermore, the saturated fatty acid methyl esters for BSF larval biodiesel (85.89%) were found to be higher than several other larval biodiesel products.

By meeting the international standards, the study underlines the feasibility of conversion of low-cost organic waste to valuable biodiesel using BSF larvae as an alternative renewable feedstock.

Leong et al. explore the potential of producing biodiesel using lipids from BSF larvae too. Three types of organic wastes (sewage sludge, fruit waste and palm decanter cake from oil palm mill) are selected and the growth rate of the larvae is determined by studying the changes in the biomass per day. BSF larvae fed with fruit waste and palm decanter cake have shown growth rates of  $0.52 \pm 0.02$  and  $0.23 \pm 0.09$  g/ d, respectively. No positive sign of growth is observed in the larvae fed with treated sewage sludge.

Biodiesel as fatty acid methyl ester is synthesized by transesterification of the larvae lipid using sulphuric acid as catalyst in methanol. Biodiesel produced was analysed using ATR-FTIR spectroscopy and GC-MS. The main compositions of fatty acid were found to be lauric acid, palmitic acid and oleic acid. Fatty acid composition of the lauric acid fed with fruit waste, sewage sludge and palm decanter was found to be most abundant in the larvae lipid. The amount obtained was 76.13%, 58.31% and 48.06% in biodiesel from fruit waste, sewage sludge and palm decanter respectively. In addition, fatty acid of palmitic acid was attained at 16.48% and 25.48% fed with sewage sludge and palm decanter, respectively. However, the authors that the quality of the biodiesel produced will be determined and optimized in future studies.

In another research, Jung et al. considered a direct conversion of BSF larvae into biodiesel through non-catalytic transesterification. In particular, they first extracted lipid in BSF larvae grown on food waste by liquid/liquid extraction, and the extract was converted into biodiesel (93.8 wt% yield) at 65 °C for 8 h by base-catalysed transesterification. Then they performed a non-catalytic transesterification of the extract of BSF larvae. This process showed 94.1 wt% of biodiesel yield after 1 min of reaction at 390 °C in the presence of a porous material (SiO<sub>2</sub>)<sup>5</sup>. The non-catalytic

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<sup>5</sup> To enhance the contact time of both reactants, the transesterification reaction was performed using a porous and non-reactive material, silica. The role of porous material is to provide the confined space of gas phase MeOH and liquid phase lipid, that can significantly increase their collision frequency (Jung et al.).

reaction was also used for direct conversion of dried BSF larvae into biodiesel without lipid extraction.

For biodiesel production from BSF larvae grown on food waste, both base-catalysed and non-catalytic transesterifications of BSF larvae extract were considered. Major fuel compositions from both processes were lauric acid, palmitic acid and ceramide, and biodiesel yields were nearly identical. However, what changed is the kinetic of the reaction. The base-catalysed transesterification was performed for 8 h, while non-catalytic transesterification rapidly converts MeOH and lipid into biodiesel (1 min), with no need of catalyst: this confirmed the superiority of direct conversion of dried BSF larvae through non-catalytic reaction in terms of time needed for the reaction to occur and environmental issue. In fact, for the base-catalysed reaction, high quantity of catalysts such as KOH and NaOH is required, resulting in inevitable generation of alkaline water into the environment. Moreover, during the wastewater treatment process, a large quantity of clean water is required to neutralize the contaminated water. For example, about 1 L of clean water is required per production of 1 L biodiesel. In non-catalytic reactions this does not happen, resulting in a more environmentally beneficial solution. In addition, the non-catalytic reaction worked for direct transesterification of BSF larvae without lipid extraction, which is desirable in terms of overall process efficiency. However, the high reaction temperature required for non-catalytic transesterification is a significant concern at the current level of technical development. As such, the author suggests that the investigation of other porous materials allowing non-catalytic transesterification at lower temperature could be considered the promising future study.

In Table 5 various parameters are considered to describe the fuel property of BSF larvae-derived biodiesel compared with Korea/U.S./EU standards.

The authors states that all fuel properties of BSF larvae measured in this study satisfied the Korea and EU biodiesel fuel standards and that non-catalytic transesterification could be considered a promising technical platform for conversion of food waste into biodiesel.

*Table 5 Fuel property of BSFL-derived biodiesel in comparison with Korea/US/EU standards*

<b>Properties</b>	<b>Value</b>	<b>KS M 2965</b>	<b>ASTM D6751</b>	<b>EN 14214</b>
Water content (mg/kg)	200	<500	<500	<500
Flash point (°C)	131	>120	>130	>120
Kinematic viscosity at 40 °C (mm <sup>2</sup> /s)	4	1.9-5.0	1.9-6.0	3.5-5.0
Density at 15 °C (kg/m <sup>3</sup> )	880	860-900	-	860-900
Acid value (mg KOHg <sup>-1</sup> )	0.12	<0.5		
Methanol (wt.%)	<0.01	<0.2		
Total glycerol (wt.%)	0.03	<0.24		<0.25

Finally, biofuels can be prepared from edible biomass; however, this is generating food versus fuel debate among the members of civil societies and the competition between using lands for growing crops for biofuel production and for food consumption is a major concern. Therefore, to address the land usage competition issue, there are needs to synthesize biofuels from nonedible waste materials and BSF larvae fed with food waste could be a possible solution.

### *1.3.3 Soil composting*

It's widely known that the intensive use of chemical fertilizers has caused huge environmental and health issues. The residue generated by the BSF during bioconversion of organic waste is considered a suitable crop fertilizer. In particular, Choi et al. state that the compost produced by the insect is equivalent to a commercial fertilizer in quality. In their study, experimental household organic waste was processed by BSF and the chemical composition and microbiota were analysed to evaluate the quality of the fertilizer. The results show that there is virtually no difference in the amount of chemicals between commercial and BSF derived compost. This indicates that the latter can be utilized immediately. The BSF derived compost just contained slightly more electrical conductivity, which may be caused by the relatively high amount of sodium. Another important feature is that the BSF derived compost based on food consumable by human is free of heavy metal. However, the chemical composition of the compost is surely subject to that of the food waste the insect consumes. In the sense of quality control, this potential problem can be solved with pre- or post-processing of the food waste and the compost.

### *1.3.4 Chitin*

Chitin is the second biggest available biopolymer on earth, next to cellulose. The polymer consists of a mixture of mainly N-acetyl-D-glucosamine and a small amount of D-glucosamine and can easily be converted to chitosan by deacetylation. Chitosan has an increased solubility in an acid environment which facilitates further processing into marketable products (Soetemans et al., 2020). Applications for chitosan include water purification and wastewater treatment, food and beverages, cosmetics, bioplastics, pharmaceutical, agrochemistry and many others, known and unknown ("A Worldwide Market with a Strong Demand"). Chitosan acts as a coagulant for both organic and inorganic matters present in water. Its purifying effect is also used by dosing it prior to filters when purifying water. It is also a very good chelating agent for heavy metals present in industrial waters. Due to its full biodegradability, non-toxic and natural origin, and owing to the growing needs in water treatment and water purification, chitosan market share in this application

is expected to grow significantly with the next years to come. Chitin and chitosan are key products, with virtually no production in Europe: in fact, most of the production comes from Asia, and is issued from shrimp and crab shells.

To sustain the growing demand of chitin and the exploration of new applications, there is a search for more and new chitin sources. BSF chitin, a by-product of the food bioconversion process, can be an effective alternative.

Chitin comes from the husks of BSF larvae and many methods of isolation have been developed in recent years. Depending on the raw material from which chitin is isolated, there are various undesirable components present in the material that have to be removed. In the case of insects, chitin is present in the chitin–melanin complex, in fungi in the chitin–glucan complex, in crustaceans, chitin forms a network with proteins where calcium carbonate is deposited. There is no one standard method of isolating chitin: different chemical (using various reagents) as well as biological (enzymatic deproteinization, fermentation) methods are used. Therefore, it is best to experimentally optimize the chitin isolation method based on the raw material used (Złotko et al., 2021). Złotko et al. extracted chitin from *Hermetia illucens* pupal exuviae using different chemical methods and they obtained a chitin yield of 5.69–7.95% which is comparable with other insect-specific studies.

Finally, researchers from WMG at the University of Warwick have successfully extracted chitin from the husks of BFS too, considering it a new economic opportunity (“Chitin Can Be Successfully Extracted from Black Soldier Fly Husks”).

#### **1.4 Use of BSF for wastewater treatment**

The use of BSF is well known for playing a crucial role in solving issues linked with high volumes of organic wastes distributed all over the world. It has progressively been employed in treating biological waste as it is seen as being an environmentally friendly and inexpensive process. However, the very first study using BSF larvae on wastewater treatment is the one of Popa and Green, 2012. In particular, they tested larvae behaviour on leachate and the authors concluded that larvae were able to reduce COD and VFAs while growing within a liquid medium.

Then, Grossule and Lavagnolo (2020) considered the adaptability of BSF larvae to leachate treatment, testing at lab-scale different leachate concentrations (25%, 50%, 75%, 100%) and two different feeding substrates: liquid (pure leachate) and semi-solid (wheat bran mixed with leachate). In all tests mortality was less than 50% and it was mainly linked to food shortages: the higher the nutrient content in leachate, the higher the larval development. Figure 4 summarizes the

results regarding the variation of average larval wet weight and the mortality. From the prepupal development, it was attested that the positive effect produced by the leachate with no signs of inhibition effects.

The authors draw the following conclusions:

- concentration and profile of lipids and proteins are both influenced by feeding substrate and might be controlled by mixing leachate with different solid substrate;
- Dry mass characterisation demonstrates that BSF prepupae biomass could be exploited as an alternative energy source in the production of biodiesel;
- among the two substrates conditions the semi-solid one proved to better perform than the liquid one.

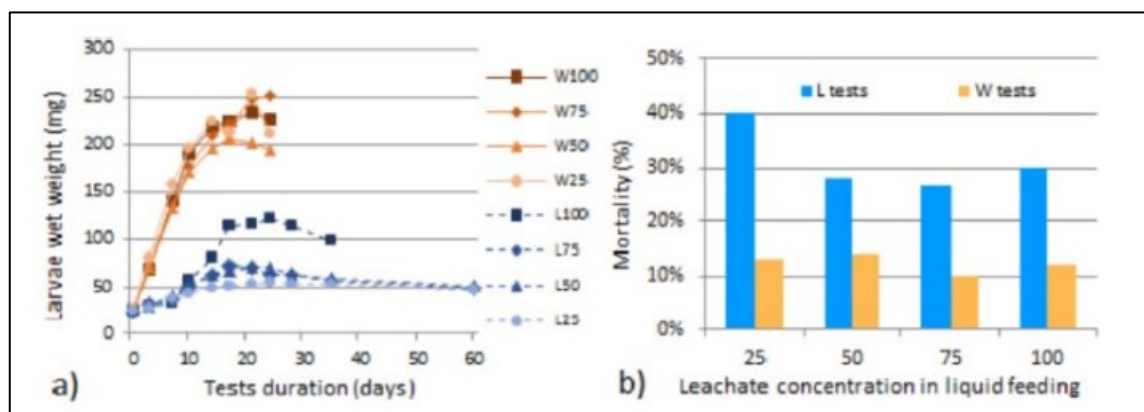


Figure 4 a) Variation of average larval wet weight under the different feeding conditions adopted (W=wheat bran substrate, L=liquid substrate, 25-100=percentage of leachate in the feeding liquid). b) Larvae mortality (% of dead larvae over the total number of larvae at the start) under the different feeding configurations. (Grossule and Lavagnolo, 2020)

After the assessment of the adaptability of BSF larvae to landfill leachate, further lab studies were carried out aiming testing alternative solid substrates, selecting preferably residues, such as wheat bran, brewers' spent grain and sawdust, in order to render the semisolid feeding approach economically advantageous and to investigate the treatment capacity of BSF larvae under various operative conditions. This step was performed in the study of Grossule et al. (2020). During the test it was monitored: larvae growth rate, in terms of weight variation, mortality and time to reach the prepupal stage; prepupal biomass composition, in terms of crude protein, lipids and fatty acids, substrates, at the beginning and the end of tests for total solids (TS), total organic carbon (TOC), total Kjeldahl nitrogen (TKN), ammonia and the 7-day Respirometric Index (RI7). The best performance was observed with wheat bran and brewers' spent grain: after only 21 days it was recorded an average larvae weight for wheat bran of  $226.1 \pm 5$  mg (w/w) with a prepupation of

more than 80%. The initial TS, TOC and nitrogen content in feeding substrates had been metabolised (gasified and accumulated in prepupal biomass) by approximately 55%, 60% and 48%, respectively. Since the focus of the study was the treatment capacity of the BSF larvae, the removal efficiencies were detected for both the larvae boxes and control boxes, they were dependent on carbon and nutrient availability. As it can be seen in Table 6, the removal efficiencies in each reactor containing larvae are higher compared to the one in the respective control reactor. TOC removal efficiencies observed for wheat bran and spent grain tests were respectively around 60% and 53%, while for sawdust test the value remained below 25% due to the low biodegradability of the support material.

*Table 6 Total solids (TS), total organic carbon (TOC), total nitrogen (TN), organic nitrogen (Norg) and 7-day Respirometric Index (RI7) reduction efficiency in 'larvae box' and in 'control box' (Grossule et al, 2020)*

Tests	Parameters	Input loads (mg/larva)	Removal efficiencies	
			Larvae box (%)	Control box (%)
W	TS	411.3	60.1	45.7
	TOC	212.3	63.8	51.4
	TN	14.2	47.8	11.7
	Norg	13.2	73.2	43.3
	RI <sub>7</sub>	--	83.5	56.4
B	TS	411.3	53.0	38.2
	TOC	231.1	59.8	44.6
	TN	19.2	48.1	26.3
	Norg	18.3	85.6	56.5
	RI <sub>7</sub>	--	83.6	73.7
S	TS	411.3	26.3	14.5
	TOC	200.0	24.4	12.9
	TN	1.4	84.2	81.6
	Norg	0.4	98.6	91.5
	RI <sub>7</sub>	--	36.5	13.5

Dry mass characterisation displayed a significant content of fats and proteins and the analysis demonstrated that *Hermetia illucens* prepupae from wheat bran and spent grain were suitable to produce biodiesel with the quality requirements established by European standards for biofuels (UNE-EN 14214); however, the potential use of proteins as an animal feed needs further studies for assessing the presence of contaminants. The main critical issues resulting from this study were:

- Relatively high mortality occurring under pure liquid condition;
- Fluctuability of leachate quality limiting full comprehension of the process.

The first was solved by appropriate set up of the reactor system, providing efficient physical support for larvae mobility (Grossule and Cossu, 2021), while the second was solved by adopting artificial leachate purpose-designed to simulate real leachate and guarantee reliable, constant,

repeatable and representative concentrations (Grossule et al., 2021). For the scaling up of the process and proper designing of BSF larvae reactor for use in HOC wastewater treatment, organic removal kinetics should be defined. In this sense, Grossule et al. (2022) provided a better understanding of the influence of the biodegradability and degree of oxidation of organic content on leachate treatment performance. Six leachates characterised by similar COD, but different BOD<sub>5</sub>/COD and TOC/COD ratios were tested. Then, the BOD<sub>5</sub>/TOC ratio was introduced to consider the effect of both biodegradability and oxidation degree. The main conclusion was that process treatment performance was highly influenced by the quality of organic substances. Higher biodegradability and lower oxidation degree resulted in a greater and faster larvae growth, with final wet weight of between 49.2 and 91.9mg/larva; lower mortality between 5 and 32%; higher prepupation percentages ranging from 4 to 21% and higher specific substrate consumption rate with values varying from 0.051 to 0.063 mgTOC/mg larva/d. As seen in Fig. 5, Yield was calculated during the growth phase until the 35th day. At the 6th day, when maximum growth rate occurred, Y varied significantly between the different tests, attesting the occurrence of a higher conversion of substrate into new biomass proportional to higher BOD<sub>5</sub>/TOC values. After the 6th day, yield decreased in all tests, approaching the same final values at the end of the growth phase.

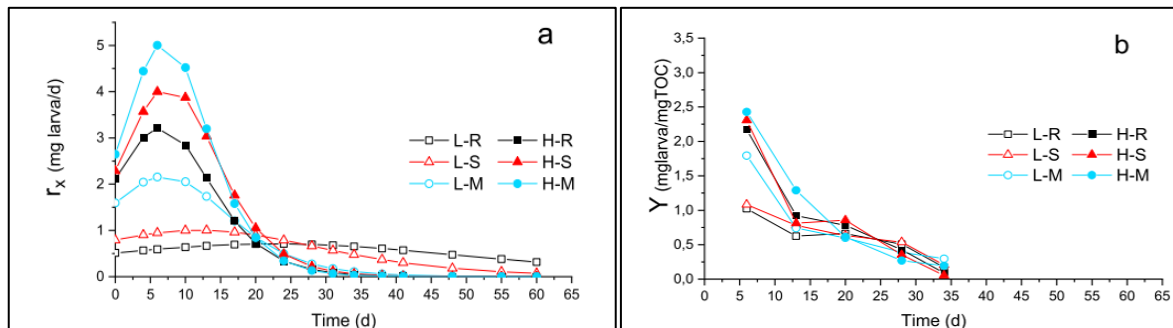


Figure 5 Variation of growth rate  $r_x$  and yield  $Y$  (b) under different leachate feeding conditions. (R = Real leachate; S=Synthetic artificial leachate, M = Mixed artificial leachate; H = high biodegradability; L = low biodegradability) (Grossule et al, 2022)

However, no significant differences were detected in larvae protein and lipid contents, including the profiling of fatty acids.

The Grossule et al. (2020) study had another interesting conclusion: the load of TOC and TN in the sawdust substrate was insufficient to support larval development, so in further studies the concentration and amount of leachate should be increased when using solid supports with low or no nutrient content. This issue was investigated in the study performed by Grossule et al. (2022), where larvae were fed with three artificial wastewaters characterised by different organic

concentrations and each type was tested at four loads. Treatment performance was assessed by monitoring larval weight variation, mortality, prepupation, and organic substrate consumption (measured in terms of Total Organic Carbon, TOC). Figure 6 shows the variation over time of average larval wet weight and substrate concentration observed with the different types of wastewater and larval densities. Larval starvation was observed in all tests when TOC concentrations dropped below 1000mgC/L and below the substrate load of 10 mgC/larva, substrate concentration in the feed did not influence larval growth. Conversely, above 10 mgC/larva the higher the load, the higher the positive impact of the substrate concentration. On the contrary, the specific substrate consumption rate appeared not to be influenced by substrate concentration but only by the organic load, considering a Michaelis Menten like relationship.

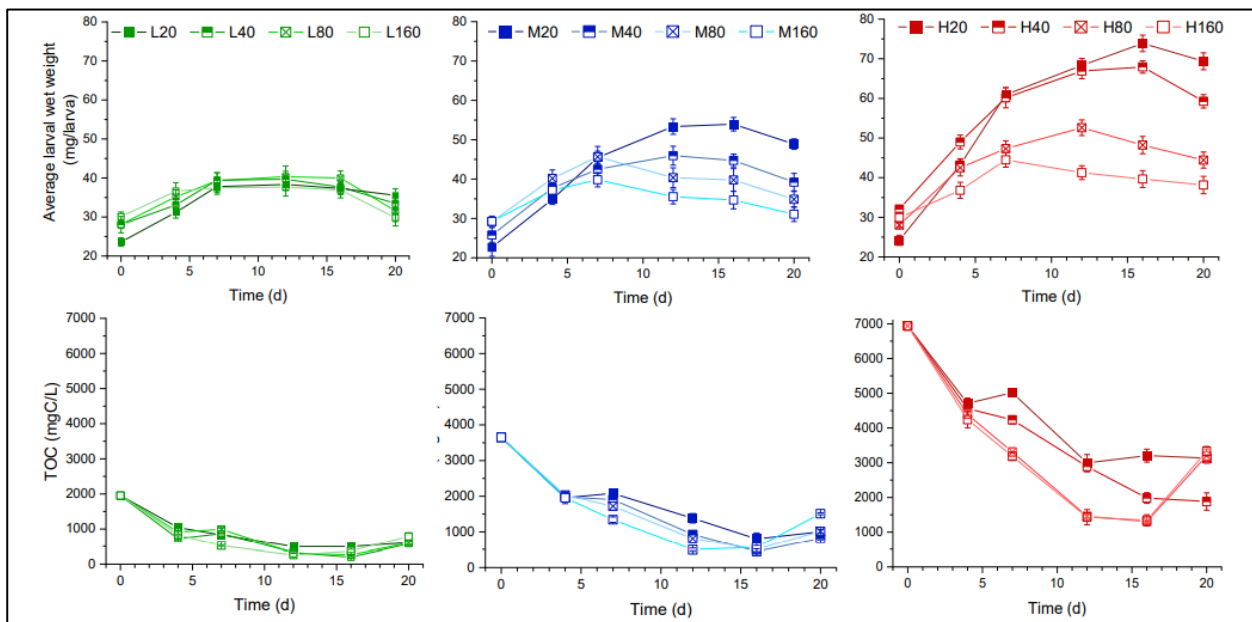


Figure 6 Variation of average larval wet weight (w/w) and substrate concentration (measured as TOC) under different leachate feeding typologies (L=low-, M=medium-, H=high-organic content concentration) and larval densities (20, 40, 80, 160 larvae per reactor) (Grossule et al, 2022)

The conclusion of the study stated that substrate load can be assumed as a design parameter for BSF treatment process, while substrate concentration might only influence potential resource recovery from larval biomass. From these reported studies, the patent regarding the use of Black Soldier Fly larvae in wastewater treatment has been filed.

However, in all these studies there's something missing: a solution for scaling up the process. Therefore, a further study tried to fill this missing step from the patent to the real scale application by the definition of the type of the physical support and the larvae density. The research focused on achieving a better knowledge of the optimal operational conditions in terms of supporting

material and larvae density, to maximise the liquid volume per unit of reactor volume and the organics removal per unit of reactor surface without compromising survival rate of larvae and maximising the treatment performance. The experiment was performed by placing young BSF larvae in batch reactors. Larvae were physically supported by three different inert supporting materials which were fully saturated (Valox®, Kaldnes® and Geomat). Four larvae density (4, 8, 16 and 32 larvae/ cm<sup>2</sup>) were tested with the same artificial wastewater in the different supports, under same organic load. Based on the results obtained, larval growth, in terms of overall larval wet weight, mortality prepupation and yield, was significantly negatively influenced by high larvae density (32larvae/ cm<sup>2</sup>), regardless the supporting material. At same organic load, the variation of the average larval wet weight appeared similar in the three larvae densities 4, 8 and 16 larvae/ cm<sup>2</sup> regardless the solid support. The only exception was test with Valox material and 16 larvae/cm<sup>2</sup> where variation of average larval wet weight was faster and greater, similarly to all tests with larvae density of 32 larvae/cm<sup>2</sup>. This is justified by the significant mortality occurred and the consequent higher amount of organic load available to survived larvae. Prepupation and mortality generally decreased with the increase of larvae density, and among the solid supports the highest prepupation and survival rate were detected in Kaldnes tests. The highest mortality and lowest prepupation occurred instead in geomat tests. Regarding the yield parameter it was observed that the greater the larvae density the higher the conversion efficiency of substrate into new biomass till the limit larvae density of 16 larvae/cm<sup>2</sup>.

### **1.5 Research objectives**

It has been seen in the previous chapter that the use of Black Soldier Fly larvae in biological treatment processes is a highly promising technique for the treatment of high organic content (HOC) wastewater.

In this study, a further step is taken, considering food processing wastewater treatment by BSF.

In particular, the wastewater streams tested are from the following food-processing industries:

- Bakery (BA)
- Brewery (BR)
- Dairy (D)
- Juice production (J)
- Slaughterhouse (S)
- Winery (W).

The aims of the study are to evaluate the compatibility of larvae with tested wastewaters, measure the removal rates of organic substance (mg/day/larva) for different wastewater streams and compare them to previous studies results.

## **2. MATERIALS AND METHODS**

### **2.1 Research program**

The experiment was performed placing in plastic boxes young BSF larvae (6-day-old, 8.4 mg as average wet weight per larva, SE 1.08). Larvae were physically supported by a plastic granular bed, which was fully saturated by the feedings (Grossule et al., 2021 and 2022).

In order to have different organic content concentrations, six different types of wastewater were considered. In this study process performance was investigated in the treatment of real wastewaters from different food-processing industries, such as Bakery, Brewery, Dairy, Juice production, Slaughterhouse and Winery.

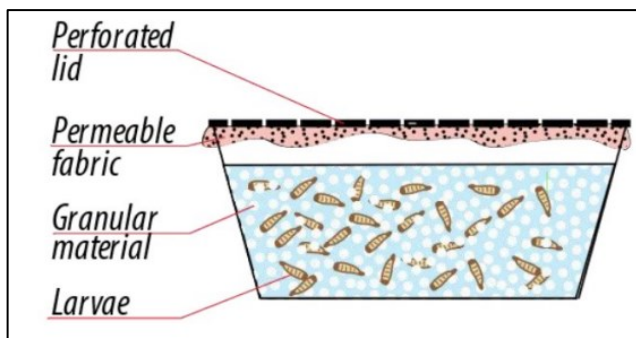
Each test was tested with a load of 80 larvae and conducted in triplicate.

### **2.2 Equipment and growth conditions**

The 21 testing reactors (Fig. 7) were made of a plastic box (13.5cm x 13.5cm x 5.5cm), containing a granular plastic bed (VALOX®, 2-3 mm diameter) (Fig. 8) completely saturated with 150mL of feeding.

The granular material allowed to larvae free movement in the liquid substrate to let them feed and breathe. (Grossule et al., 2022)

Each box was covered by a permeable non-woven fabric to avoid the escape of larvae and prevent the intrusion of other species and by a perforated plastic lid to allow air recirculation. All tests were carried out in a thermal insulated room under the same environmental conditions suggested by Grossule and Lavagnolo (2020): temperature range 25-30 °C; photoperiod Light/Dark of 18/6h.



**A**



**B**



**C**

Figure 7 Testing reactor: scheme (A) (Grossule et al, 2022), Top view (B) and front view (C) of the granular support bed mixed with wastewaters.



Figure 8 Support material, VALOX®

### 2.3 Feeding, operation and monitoring

At the beginning of the experiment, 150 mL of feeding was added to each box. It was replaced every week, up to 60 days, to provide constant feeding conditions. Twice a week, 10 larvae were collected from each box and weighted individually on an analytical balance. The larvae were then returned to their boxes. At the time of feed replacement, liquid volume was measured, sampled, and analysed total organic carbon (TOC), chemical oxygen demand (COD), biochemical oxygen demand (BOD5), total Kjeldahl nitrogen (TKN), ammonia and phosphate. Moreover, tested wastewaters were analysed in terms of carbohydrates, proteins and lipids.

Prepupae, recognised by darkening of their colour, were removed, washed, weighed and frozen. Larval development and growth were assessed in terms of variation of larvae wet weight, mortality, number of prepupae, variation of TOC concentration and wastewater volumes.

Parameters relating to conversion efficiency of substrate into new biomass and specific substrate consumption rate were calculated too.

Conversion efficiency of substrate into biomass by anabolic processes is defined by the yield (Y), expressed as mg larvae/mgTOC consumed. Yield values were calculated during the whole growth phase until the 49<sup>th</sup> day as follows:

$$Y = \frac{\Delta X}{\Delta TOC} = \frac{\text{total larvae growth weight (mg)}}{\text{total substrate consumption (mgTOC)}}$$

The specific daily substrate consumption ( $V_{TOC}$ , mgTOC/larva/day), was introduced as a simple parameter and calculated over the whole testing period according to the following equation:

$$V_S = \frac{dS}{X_0 dt} = \frac{S_0 - S}{X_0(t - t_0)}$$

where:

$S_0, S$  = substrate mass at the beginning and end of the monitoring period, measured as TOC;

$S_0 - S$  = removed substrate (mg TOC);

$X_0$  = initial number of larvae or initial larvae wet weight (number of larvae or mg) at time  $t_0 = 0$ ;

$t$  = monitoring time (days).

The same concept was applied for the specific daily nitrogen consumption ( $V_N$ , mgN/larva/day) and the specific daily phosphorus consumption ( $V_P$ , mgP/larva/day).

As suggested by Grossule et al. (2023), relationship between  $V_S$  and organic loads should fit a Michaelis Menten like relationship:

$$V_S = \frac{V_{S_{max}} \times TOC_{load}}{K_S + TOC_{load}}$$

Where:

$V_S$  = specific substrate consumption rate (mg TOC/larva/d)

$V_{S_{max}}$  = max specific substrate consumption rate (mg TOC/larva/d)

$K_S$  = half saturation constant (mg TOC/larva)

$TOC_{load}$  = TOC load (mgC/larva).

## 2.4 Analytical procedure

Wastewater streams were analysed using Standard International Methods for the following parameters: pH, TOC, COD, BOD<sub>5</sub>, ammonia nitrogen, Total Kjeldahl Nitrogen, Total Phosphorous. TOC was measured using a TOC-VCSN Shimadzu Analyzer; COD was determined by a photometric method; BOD<sub>5</sub> were determined according to the standard Italian method IRSA-

CNR (29/2003 vol 2 n. 5130; 29/2003 vol 2 n. 5120 B2). Ammonia nitrogen was measured with a distillation-titration procedure and TKN was measured by means of a distillation-titration procedure after an acid digestion phase. Total phosphorus (after sample digestion) was determined using a UV–vis spectrophotometer (Shimadzu UV-1601). Larvae were weighted using an analytical balance. VFA was measured by the FOS/TAC procedure.

Lipids extraction was performed via Soxhlet method with petroleum ether, after acid hydrolysis. Lipid extracted was subject to transesterification and later fatty acids identified by Gas chromatography (GC) and FID.

Finally, carbohydrates were injected in HPLC Refractive Index Detectors (HPLC RI Detectors)

### 3. RESULTS AND DISCUSSION

#### 3.1 Wastewater quality

Table 7 summarizes the characteristics in terms of classic parameters of the wastewater streams used in this study.

*Table 7 Initial wastewater streams' quality data in terms of classic parameters*

	<b>BA</b>	<b>BR</b>	<b>D</b>	<b>J</b>	<b>S</b>	<b>W</b>
<i>pH</i>	4.6	7.7	9.2	5.8	7.3	5.5
<i>TOC (mgC/L)</i>	1902	1566	358	744	433	1842
<i>COD (mgO<sub>2</sub>/L)</i>	16200	6450	2650	4200	2750	8950
<i>BOD<sub>5</sub> (mgO<sub>2</sub>/L)</i>	6315	2572	1264	1123	1027	4564
<i>TKN (mg/L)</i>	250	199	31	24	139	89
<i>NH<sub>4</sub><sup>+</sup> (mg/L)</i>	50	56	6	<5	46	46
<i>N – Organic (mg/L)</i>	200	143	25	19	93	43
<i>P<sub>tot</sub> (mg/L)</i>	4	10	10	<0.7	14	16
<i>BOD<sub>5</sub>/COD</i>	0.39	0.40	0.48	0.27	0.37	0.51
<i>TOC/COD</i>	0.12	0.24	0.14	0.18	0.16	0.21
<i>BOD<sub>5</sub>/TOC</i>	3.32	1.64	3.53	1.51	2.37	2.48

Considering the parameters representing the organic carbon content in the feedings, the highest values in terms of both TOC and BOD correspond to BA, BR, W and L. On the contrary, for D, J and S the table shows a low organic content.

Further to quality parameters, the BOD<sub>5</sub>/TOC ratio was used: its definition derives from the ratio between degrees of Biodegradability and Oxidation:

$$\frac{BOD_5/COD}{TOC/COD} = \frac{BOD_5}{TOC}$$

BOD<sub>5</sub>/TOC may be considered a Biotreatment Index (BI): the higher the value, the higher the concentration of BOD and the lower the degree of oxidation of organic content in the specific liquid. In broad terms, BOD can be still used as a monitoring parameter for larvae, but, at the beginning, it was used as a wastewater treatment index when dealing with activated sludge. Thereby, it can give rough information about the substrate biodegradability, but in order to have a more detailed analysis of the larvae treatment it's not an optimal parameter. Instead, the key parameter which makes the difference, when using complex organisms such as larvae, is the composition of the BOD value in terms of macronutrients. Table 8 shows the wastewater streams' nutrients composition in terms of proteins, carbohydrates, and lipids, while Table 9 shows the quality data in terms of Volatile Fatty acids (VFAs).

*Table 8 Initial wastewater streams quality in terms of nutrients composition.*

*C:D = total amount of Carbon atoms: number of Double (unsaturated) bonds.*

	BA	BR	D	J	S	W
<i>Proteins (mg/l)</i>	1562.5	1243.75	193.75	150	868.75	556.25
<i>Carbohydrates (mg/L)</i>	624	4	15	1618	156	15
Starch (%)	8.8	100.0	100.0	32.9	96.2	100.0
Glucose (%)	-	-	-	11.2	3.8	-
Sucrose (%)	-	-	-	15.5	-	-
Fructose (%)	91.2	-	-	40.4	-	-
<i>Lipids (mg/L)</i>	2476	249	745	24	497	68
Myristic acid C14:0 (%)	0.5	1.9	11.7	5.2	3.0	3.9
Palmitic acid C16:0 (%)	11.7	37.1	34.8	30.6	28.5	30.6

Palmitoleic acid C16:1 (%)	0.4	0.8	1.6	2.2	1.3	5.9
Stearic acid C18:0 (%)	6.7	36.4	10.3	12.4	28.9	13.8
Olenic acid C18:1 (%)	67.5	2.4	22.1	18.9	23.4	6.1
cis-Vaccenic acid C18:1n7 (%)	0.1	0.8	0.7	4.9	3.7	8.2
Linoleic acid C18:2 ct (%)	5.5	4.1	1.1	6.8	0.6	1.2

Table 9 Initial wastewater streams quality in terms of volatile fatty acids composition

<b>Volatile fatty acids composition</b>	<b>BA</b>	<b>BR</b>	<b>D</b>	<b>J</b>	<b>S</b>	<b>W</b>
Total (mg/L)	947	753	3.88	88.6	135.3	815
<i>Acetic acid</i> C2:0 (%)	48.2	65.8	100.0	83.2	37.6	50.2
<i>Propionic acid</i> C3:0 (%)	9.2	19.0	-	7.0	20.8	5.1
<i>Butyric + isobutyric acid</i> C4:0 (%)	32.0	6.2	-	4.3	17.4	22.1

The highest presence of VFAs is found in BA, BR and W. In Figure 9 a clustered bar chart shows the amount of lipids, VFAs, proteins and carbohydrates (mg/L) present in each feeding. D, J, S and W contain a low amount of nutrients in absolute terms, compared to BA and BR.

BA contains the wider presence of nutrients, while in D and J they are significantly unbalanced. This can be better noticed in Figure 10: 100% stacked bars compare the percentage that each value contributes to the total across categories. It's interesting to notice that from the analysis it turns out that in BR and W there's an important concentration of VFAs with respect to lipids and proteins present in the same feedings.

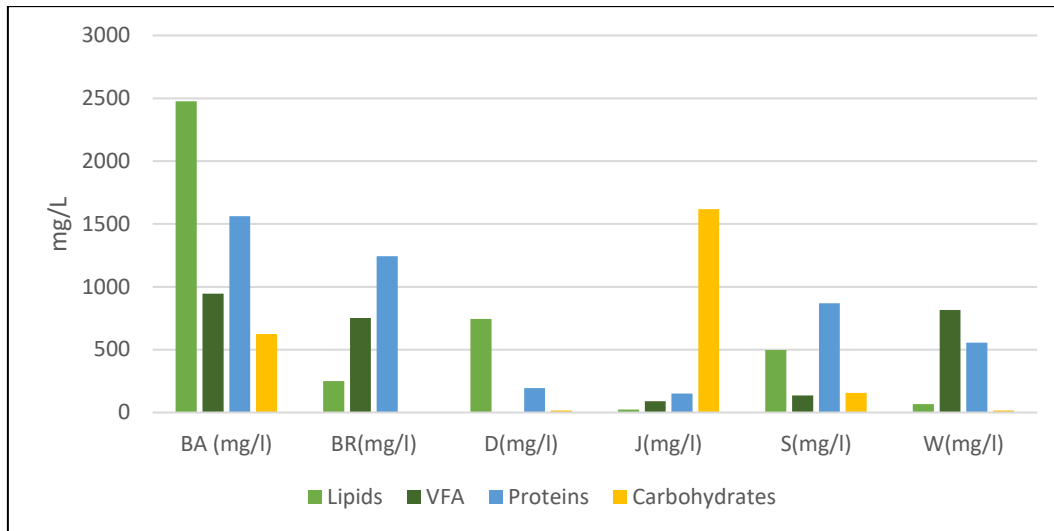


Figure 9 Lipid, volatile fatty acid (VFA), protein and carbohydrate content (mg/L) in each reactor test

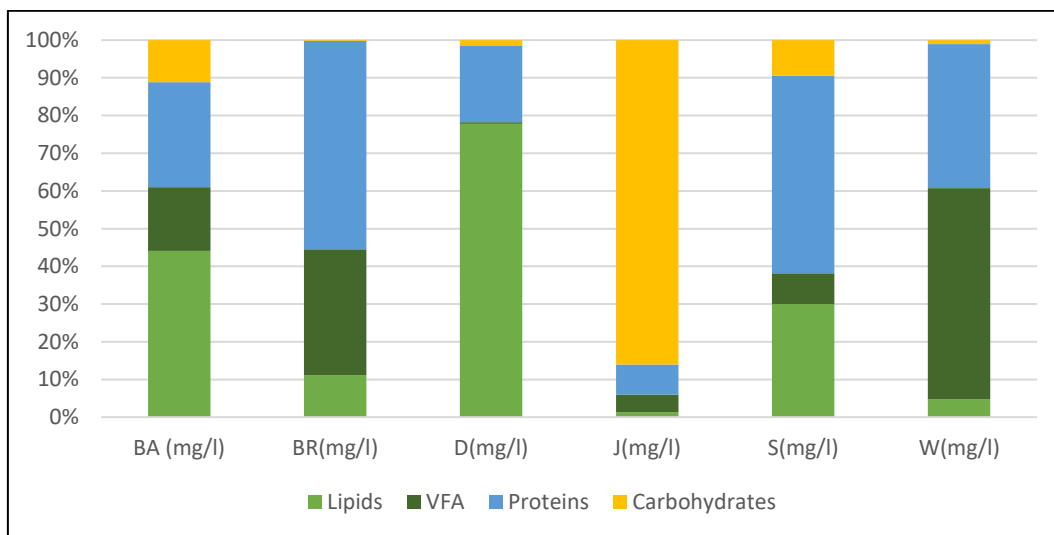


Figure 10 Lipid, volatile fatty acid (VFA), protein and carbohydrate content (%) in each reactor test

Considering the fact that carbohydrates are assimilated by larvae as fatty acids as mentioned above, in Table 10 the protein:(carbohydrates + lipids + VFAs) ratios are shown.

Table 10 Protein : (carbohydrates + lipids + VFAs) ratios

Wastewaters	Normalisation of nutrients contents	
	P	C+L+VFAs
BA	1	2.6
W	1	1.6
BR	1.2	1
J	1	11.5
D	1	3.9
S	1.1	1

### 3.2 Larval growth

Figure 11 illustrates the variation over time of average larval wet weight (w/w) observed with the different feedings. BSF larvae grew under all different feedings, but with different patterns. As confirmation of quality data previously reported, the larvae fed with BA, W and BR displayed a faster and higher development when compared with larvae growing on J, D and S. In general, larval growth was greater and occurred faster when moving from lower to higher organic content concentrated wastewaters.

The lowest performance in terms of larval growth is shown tests conducted with J. This is linked to the diet they were subject to: in J, almost 85% of nutrients is composed by carbohydrates, in particular simple sugars. With such a low amount of proteins, larvae were destined to starve. Therefore, as expected, it's clear that there's no compatibility of larvae with the tested wastewater. When larvae have problems to adapt to a certain feeding, there's a great larvae's selection which leads just to the strongest ones to remain alive. This is demonstrated both by the high mortality and the fact that in 49 days larval weight has not increased significantly.

S and D test showed a low growth performance too. In the case of S tests, as seen in the previous chapter, probably the unsuccessful growth of larvae was due to the low organic content: in fact, even if the diet was balanced in terms of nutrients, their concentrations were extremely low.

In D tests, besides the fact that the feeding was particularly diluted, thus nutrient concentrations were too low to give larvae the possibility to develop, the diet itself was unbalanced: as cited in chapter 1.2, Gold et al., 2020 suspected that excess lipids can obstacle and may decrease larval development.

Larvae fed with BR and W feedings had a great larval growth. In this case it's clear how the BOD<sub>5</sub> is a misleading parameter: besides the fact that BR had the lowest BOD/TOC ratio with J, larvae developed successfully. Thereby, it's important to focus on the amount of organic content, which was high in both BR and W, and the nutrients concentrations. If looking just on lipids, proteins and sugars, the tendency would be to think that BR and W are not characterized by a balanced composition of nutrients, because of the prevalence of proteins. However, high concentrations of VFAs were found in both feedings and there is probably a high amount of ethyl alcohol too, naturally present in brewery and winery wastewaters. In BA its presence could be possible thanks to the first step of a degradation process started when wastewaters were probably stored in anaerobic conditions before being sent to treatment. Considering both the great larval growth and the concentrations of VFAs, it's clear that larvae preferred short-chain fatty acids, in particular acetic acid, as shown in Table 9: the greatest increase in larval weight happened in BA, BR and

W, were VFAs were present the most. This observation seems logical: larvae prefer to feed on easily digestible food, instead of long chain fatty acids.

BA test is the one reaching the highest final average larval wet weight, equal to 109 mg (w/w) and the only one to enter the prepupation stadium within the test period. The counted prepupae were 102, almost 43% of the total number of larvae. When they start becoming prepupae, they lose some weight because they lose humidity, and it is clear in from Figure 9 if looking the last two weeks. Moreover, the amount of nutrients present in BA reactor tests was particularly high, giving to all larvae the possibility to feed in a balanced way. In this case, as observed by Gold et al, 2020 lipids can promote larval development too.

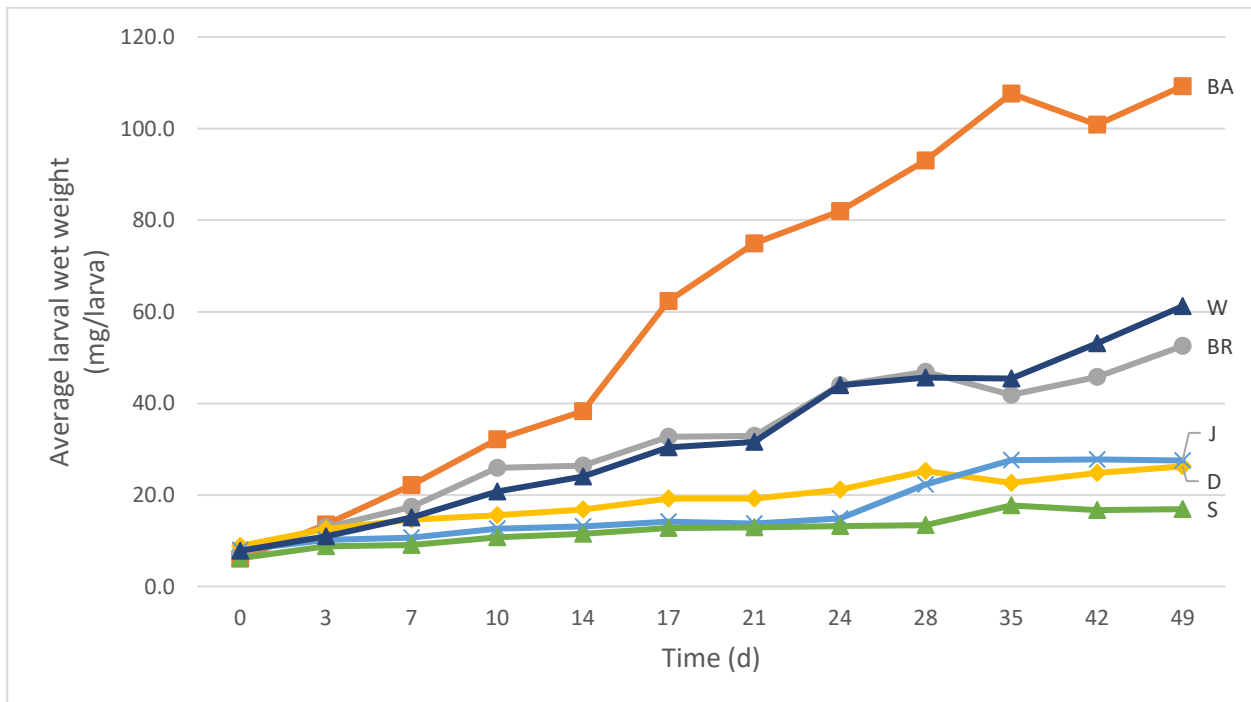


Figure 11 Variation of average larval wet weight under different wastewater feeding.

The mortality (% dead larvae over total initial number of larvae) values are shown in Figure 12. The highest mortality value was recorded by J (65%), proving that larvae were not easily adapting to the feeding. The same concept can be applied to larvae fed by S: mortality was quite high (43%). In this case the problem wasn't the adaptability to the substrate, because the diet seems to be quite balanced, but probably the fact that larvae had low concentrations of nutrients to feed on. The mortality in the other substrates was under 30% and the lowest was found in D and W (10%).

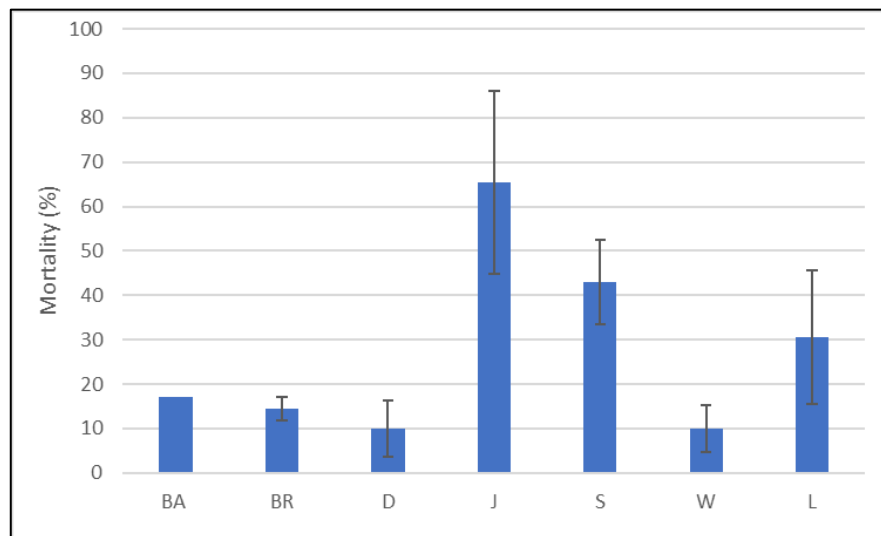


Figure 12 :Mortality (% dead larvae over the total initial number of larvae).

The yield (mglarva/mgTOC) varied significantly between the different leachates and Figure 13 attests the general trend of a higher conversion of substrate into new biomass proportional to higher BOD<sub>5</sub>/TOC values. Inevitably, it is influenced by the organic load: if the substrate is more or less diluted makes the difference in this plot. However, it's interesting to focus on the follow consideration: even if J and L have a similar BOD/TOC ratio, BR has a higher yield. This confirms again the consideration that the BOD is not an optimal parameter to use to describe complex processes as wastewater treatment by larvae is. Instead, the composition of BR in terms of typology of organic substance (macronutrients) and the organic load here make the difference.

The overall maximum yield value was achieved by D with a value of 17,3 mglarva/mgTOC.

These results revealed how, even at similar COD concentration, as for D and S, larvae metabolism appeared to be influenced by the quality of organic content of the treated wastewater in terms of nutrients, as well as degree of biodegradability and oxidation. Similar results were observed by Grossule et al. (2022), who obtained higher substrate to biomass conversion when feeding larvae with high percentages of good quality food.

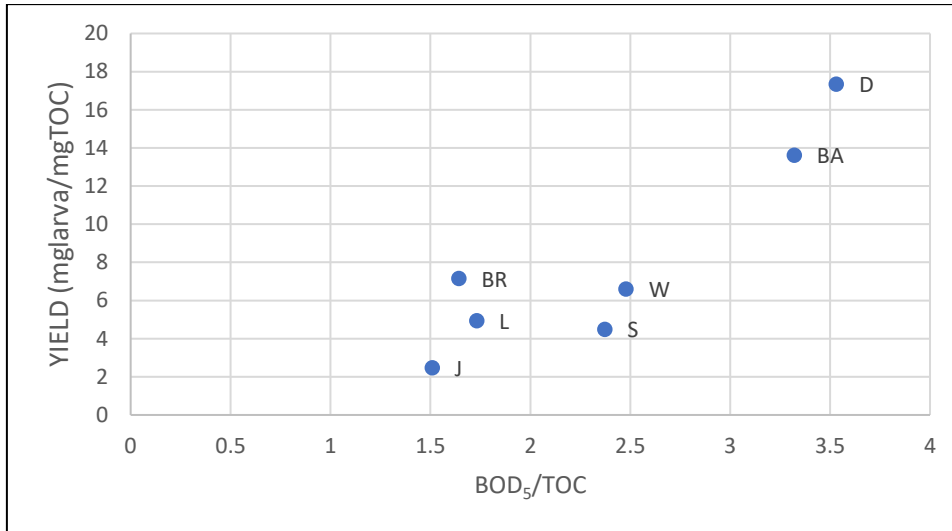


Figure 13 Variation of Yield values (mglarva/mgTOC) with BOD<sub>5</sub>/TOC ratio.

### 3.3 Substrate removal performance

The relationship between  $V_{TOC}$  and TOC loads is plotted in Figure 14.

The considerations of Grossule et al., 2023 are here confirmed: the specific substrate consumption rates ( $V_s$ , mgC/larva/day) increase nonlinearly with organic loads, regardless of substrate concentration, fitting a Michaelis Menten like relationship.

By fitting the experimental data and the Michaelis Menten relationship, the following parameters values can be obtained:

$$V_{Smax} = 2 \text{ (mg TOC/larva/d)}$$

$$K_s = 2.18 \text{ (mgC/larva)}$$

Substrate concentration influenced larval growth, and thus the potential amount of recoverable larval biomass because larvae spend much more energy to absorb diluted organic substance instead of concentrated one but had no effect on specific substrate removal rate.

From the comparison between Figures 13 and 14, it's immediate that D test boxes that have the least removal of substrate, have also the highest yield. This could be interpreted as following: if larvae feed on low organic loads, they use all of it and store it as biomass. On the contrary, if larvae are given high organic loads, as in L, W, BR tests, they do not transform it just in biomass, but in energy too. Consequently, the percentage of substrate converted into biomass is lower.

However, this general idea is not true for BA test: in fact, there, larvae got high organic load, yield and substrate consumption rates. This could be related to the quality of feeding: since each feeding has a different quality in terms of nutrients and biodegradability, it's not simple to explain each relationship and make comparisons among them. The results about BA removal performance are

confirmed by Gold et al, 2020: they observed high conversion efficiencies for feeds with a high lipid and protein content, as happens in BA test reactors.

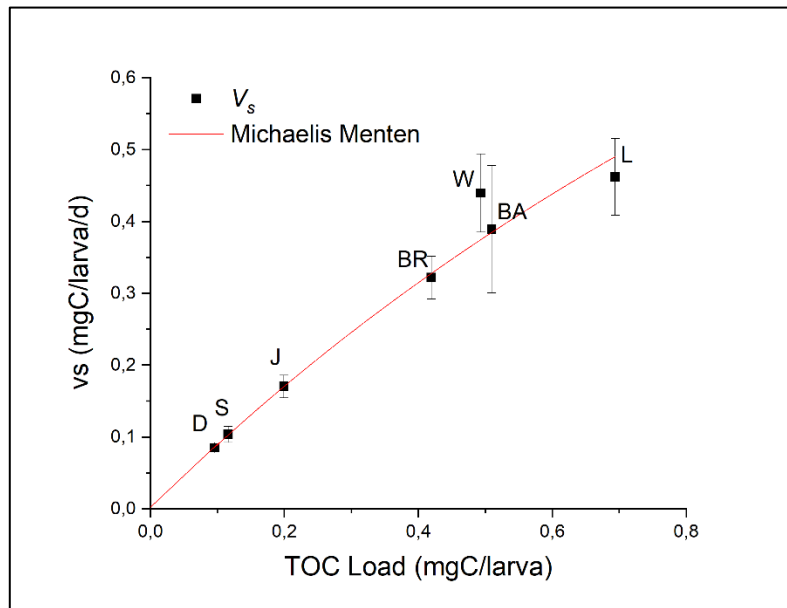


Figure 14 Variation of specific substrate consumption rates ( $V_{TOC}$ , mgC/larva/day) with TOC loads.

Figure 15 shows the variation of specific substrate consumption rates with  $BOD_5/TOC$ . It seems that the substrate quality, thus the biotreatment index, influences just how larvae store the organic substance as biomass (Fig. 12), but not how much of it they eat daily.

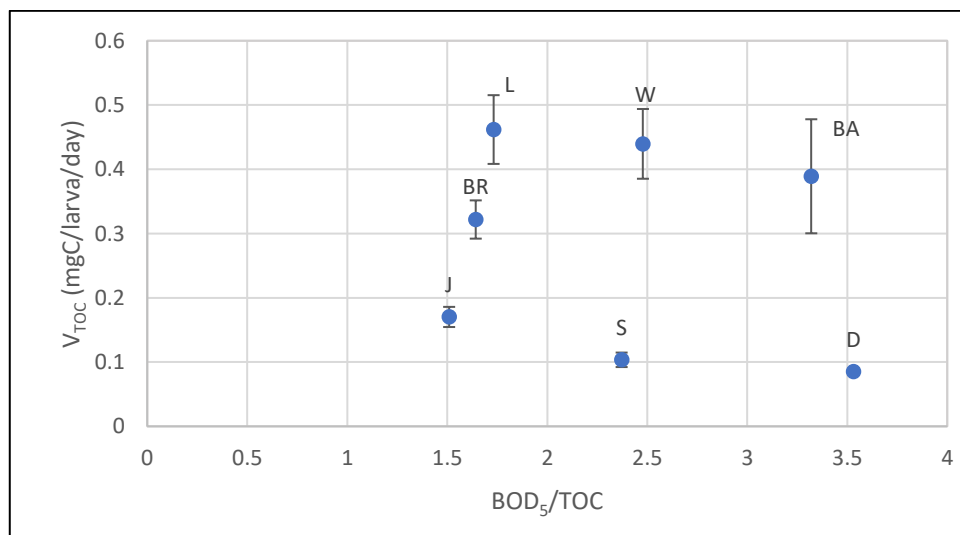


Figure 15 Variation of specific substrate consumption rates ( $V_{TOC}$ , mgC/larva/day) with  $BOD_5/TOC$  ratio.

### 3.4 Nutrients removal performance

Up-to-date studies about wastewater treatment by larvae have not considered nutrients (N and P) removal performance. In this one the objective was to understand at least the order of magnitude of daily nutrient consumption and efficiency. It was applied the same definition of the specific

daily substrate consumption, but removed Norg and phosphate were considered instead of removed substrate. They were graphically represented in Figures 15 and 16.

The line  $y=x$  represents the points of 100% efficiency, which means that all the amount of nutrient is removed from the substrate and taken by larvae. It's visible that the tendency line for Norg removal is quite close to  $y=x$ : in particular, it has a slope of 0.72. On the contrary, the phosphorus tendency line has a slope of 0.42, which indicates a poor removal performance. This could be explained by the fact that in the feedings there was an excess in nutrients, with respect to larvae's needs.

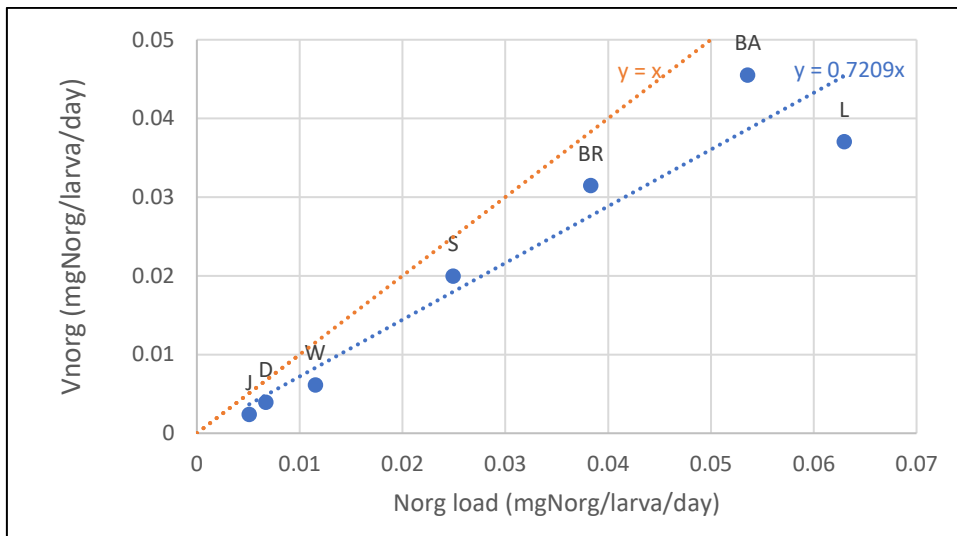


Figure 16 Variation of specific nitrogen consumption rates ( $V_{Norg}$ , mgNorg/larva/day) with Norg loads.

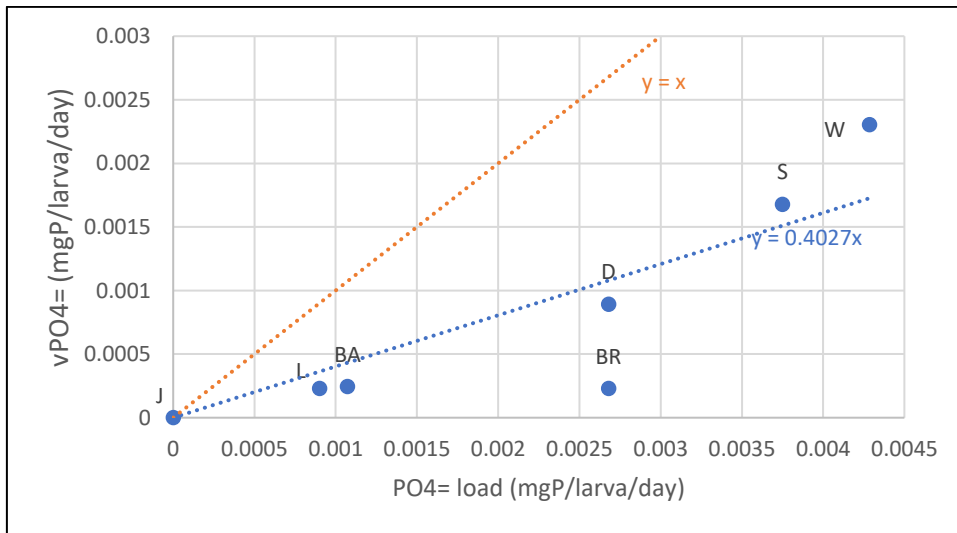


Figure 17 Variation of specific phosphorus consumption rates ( $V_{PO_4=}$ , mgP/larva/day) with  $PO_4=$  loads.

## CONCLUSIONS

The compatibility of larvae with food processing wastewaters and their substrate removal performance were investigated by monitoring larval growth in terms of larval weight variation, prepupation and mortality, and wastewater quantity and quality, in terms of both classic parameters and nutrients composition of tested feedings.

The quality of organic content in wastewaters significantly affected process treatment performance, allowing the following conclusions to be drawn based on the results obtained:

- In general, larval growth was greater and occurred faster when moving from lower to higher organic content concentrated wastewaters. J, S and D were the least concentrated feedings. Besides that, in J tests the diet was mainly composed by simple sugars and in D tests there was an excess of lipids: unbalanced diets didn't give larvae the possibility to develop. The same reasons can explain the high mortality in J and S tests.
- Larvae preferred to feed on short-chain fatty acids, in particular acetic acid, with respect to lipids: larvae that had the highest larval growth and the best substrate removal performance where those fed by BA, W and BR, where the VFAs concentration was significantly high and the diet balanced in lipids, VFAs, proteins and carbohydrates.
- In broad terms, the yield follows the general trend of a higher conversion of substrate into new biomass proportional to higher BOD<sub>5</sub>/TOC values. However, among similar BOD<sub>5</sub>/TOC values, diversities in yield values are visible due to different organic load and typology of organic substance.
- The specific substrate consumption rates increase with organic loads, regardless of substrate concentration, fitting a Michaelis Menten like relationship. Substrate concentration influenced the potential amount of recoverable larval biomass but produced no effect on specific substrate removal rate.
- The specific consumption rates of N and P generally showed a poor removal performance, probably indicating that feedings were in excess of nutrient with respect to larval need to develop.

As final conclusion, the optimal feeding among the tested ones, both in terms of larval development and substrate removal efficiency, was BA.

W, BR and S, which were balanced in diet but more diluted, could give similar results by increasing the organic load. In the case of D and J, both diluted and unbalanced, a mixture of

feedings could be the solution in order to balance the nutrients and simultaneously increase the organic load.

The overall good performance of BA, W and BR suggests that besides the good quality values of the feeding in terms of classic parameters, such as COD, TOC and BOD, the key parameter which makes the difference, when using complex organisms such as larvae, is the composition of the organic substance in terms of macronutrients.

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