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**MASTER THESIS**

# Use of Black Soldier Fly larvae for wastewater treatment: threshold of organic concentrations under various organic loads and optimum diet

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# ABSTRACT

Water is vital for all living organisms, natural cycles in ecosystems, and human existence. The availability and quality of water are crucial to maintain ecological stability. However, with increasing industrialization, urbanization, and agricultural activities, water pollution has become a major global problem. Although traditional wastewater treatment methods are effective, these approaches have notable disadvantages.

On the contrary, the use of Black Soldier Fly Larvae (BSFL) in wastewater treatment is an innovative environmentally friendly way, and also a highly efficient alternative to traditional methods. BSF larvae can convert organic waste into valuable biomass. Unlike traditional treatment methods, BSFL minimizes waste production, reduces dependence on energy-intensive technologies, and also contributes to resource recovery.

This research investigates how different Total Organic Carbon (TOC) and organic load concentrations affect the performance of BSF larvae. The aim is to determine the lowest TOC levels and ideal ranges that support larvae survival and growth and also the best removal efficiencies.

To achieve this goal, five different TOC concentrations and six different organic load values are analyzed under controlled experimental conditions. Different key performance indicators are used, such as; larvae survival, treatment efficiency, specific substrate consumption rates, biomass yield, and average wet weight. By analyzing these values, threshold values are determined to define the optimal conditions to maximize BSFL-based wastewater treatment efficiency. Overall, this study contributes to the growing body of research on sustainable wastewater treatment technologies and the potential role of insect-based bioconversion in environmental engineering.

*To my family*

## Table of Contents

## Index of Figures

## Index of Tables

## 1. Introduction

All living organisms, natural cycles of ecosystems, and human survival depend on water strictly. The availability of water and its quality are essential for sustaining ecological balance. On 28 July 2010, the United Nations (UN) recognized water and sanitation as human rights. Besides the necessity of water to maintain life, it has also an important role in the economy. This fact makes water very valuable and cannot be replaced. Over the last century, water consumption has increased dramatically due to population increase which this led to the overuse of water as a result environmental degradation has arisen and this also caused the overuse of natural resources. Water consumption by industries, domestic activities, and agriculture is getting higher and it is expected to increase to 55% by 2050 due to climate change and population growth. Specifically, Italy, Spain, Greece, Cyprus, and Southern Europe, places with high agricultural activities, are very vulnerable to this situation (Mannina et al., 2022). Moreover, even in places with an adequate amount of rainfall, some regions have started to face water scarcity. In the EU (EUROPEAN UNION), around 11 percent of the population and 17 percent of the lands have started to feel water scarcity. Also, water pollution, resulting from agricultural activities and industries is creating a big problem (Smol & Koneczna, 2021). Consequently, water quality degraded dramatically. To address these issues sustainable wastewater management and water management is very crucial. To solve these challenges, the European Commission (EC) has incorporated water management into the Circular Economy Action Plan, which is the key element of the European Green Deal. The European Green Deal (EGD) aims to make the European Union climate-neutral by 2050, and for this aim, the EC is promoting water reuse (Smol & Koneczna, 2021). In this context, a circular economy can help to reduce the usage of natural resources and decrease their environmental impacts bu reusing the wastewater. A Circular Economy (EC) is a reproductive system that is designed to increase lifespan, maximize value, and minimize waste. Due to the growing demand for water and its vital role in sustaining life the environment and economic activity, the concepts of the circular economy can be applied to water and wastewater even though they were initially applied to raw materials (Smol and Koneczna 2021). This is given priority in the 2030 Agenda for Sustainable Development by the United Nations through Sustainable Development Goal 6 (SDG 6) which focuses on addressing water scarcity and increasing water-use efficiency. The Circular Economy Plan (also known as Closing the Loop) was introduced by the European Commission (EC) in 2015 with a focus on resource recovery and reuse (Guerra-Rodríguez et al. (2020). These problems may be resolved by treating and reusing wastewater. These problems include the fact that millions of people in developing countries lack access to sanitary facilities that untreated sewage is frequently dumped into bodies of water and that sanitary and reasonably priced wastewater treatment technologies are required. By moving to closed-loop and sustainable systems these problems can be resolved by lowering pollution and enhancing public health by recovering water and nutrients that can be used again in agriculture. Closed-loop systems focus on recycling resources instead of wasting them (Jhansi & Mishra, 2013). Expanding the reuse of treated wastewater is essential for mitigating water scarcity, especially in Southern Europe (Mannina et al., 2022).

Even though conventional treatment methods for wastewater have been widely used, they have significant disadvantages. Those methods can be summarized as; chemical, biological, and physical methods. For instance, chemical treatment needs, usually, high amounts of chemicals, which makes it costly and produces huge volumes of sludge and this is also hard to handle. Similarly, physical processes are energy-intensive, which makes them costly. Also, biological treatments, including aerobic and anaerobic, have their limitations. Moreover, physical processes are usually energy-dependent, so it makes them costly also. Lastly, for biological treatment methods, aerobic and anaerobic there are also limitations such as in aerobic treatment method process needs aeration and it produces a large amount of sludge. When anaerobic is considered, it has high retention times and it produces gases like hydrogen sulfide. When integrated systems are considered, they also have drawbacks like high capital costs and operational complexities (Crini & Lichtfouse, 2018).

In contrast to traditional methods whose drawbacks are explained in the previous paragraph, the usage of Black Soldier Fly Larvae (BSFL) is an innovative, sustainable, and very promising way to treat wastewater. BSF Larvae can efficiently process organic substances to high-value biomass, such as proteins, lipids, and bio-products like biodiesel and chitin, offering significant advantages over conventional activated sludge processes.

In this method, high removal efficiency can be achieved, and it aligns with circular economy principles. BSFL can stabilize and metabolize high organic content wastewater efficiently and can achieve carbon removal up to 97 percent (Grossule, 2024). Advantages of the usage of BSFL include up to three times higher organic removal efficiency, no production of excess sludge, and the potential for resource recovery through biomass. BSF larvae have emerged as a promising biological treatment for high organic content (HOC) wastewater, such as landfill leachate and food-processing effluents. BSF Larvae convert waste into high-value biomass, which can be used in various ways, such as animal feeding and biorefineries for products like chitin, biodiesel, and antimicrobial peptides. The BSFL system offers advantages compared to traditional treatment methods. They require minimal energy and chemical inputs, produce less sludge, and reduce environmental pollution (Grossule et al., 2023).

The use of Black Soldier Fly Larvae (BSFL) offers a sustainable approach to treating leachate, which is a byproduct generated from the decomposition of organic waste. Leachate contains a wealth of organic compounds and possesses a significantly high chemical oxygen demand (COD), and the treatment of leachate through traditional methods tends to be expensive. BSF Larvae represent an innovative and promising alternative method worth considering. Furthermore, the BSFL method demonstrates a quicker processing rate and enhanced efficiency compared to standard microbial treatment techniques. Additionally, it incurs lower operational and infrastructure costs compared to conventional chemical or microbial treatment options. This approach alleviates the financial burden of waste management while also generating valuable byproducts.

BSFL usage may contribute to a reduction in  Greenhouse Gas Emissions (GHGs) since it converts organics quickly and recycles nutrients back into valuable biomass. This is a superior aspect of usage of BSFL instead of traditional treatment methods. In conclusion, the usage of BSFL transforms waste streams into valuable products instead of sending them to landfills or incineration processes. In this context, it promotes a sustainable approach by using circular economy principles.

BSFL usage illustrates this sustainable framework for multiple reasons. Firstly, waste valorization can be considered. For instance, BSFL is using the leachate to feed itself; in other words, it is a resource for itself. This means that rather than seeing the leachate only as waste, now it can be seen as a nutrient source for larvae. Additionally, BSFLs are very good at eliminating pollutants from leachate, including alcohols, volatile organic acids (VOAs), and organic nitrogen. This helps to develop more sustainable waste management practices by mitigating the risk of harmful environmental runoff.

Secondly, the process aims at nutrient recovery by converting these nutrients into valuable biomass. BSFL metabolizes nutrients and transforms them into biomass, which can be used as feed for animals or in aquaculture.

Lastly, BSFL processes are characterized by high conversion efficiency, which minimizes both material and energy loss. Also, there is a mutualistic relationship between waste treatment and product formation. Unlike methods relying on microbial treatments that primarily aim to purify wastewater, BSFL manages to process waste while concurrently producing biomass with significant commercial value. In conclusion, incorporating BSFL into circular economy systems aligns with the "waste-to-resource" philosophy. It tackles issues such as the rising volume of organic and liquid waste, resource shortages, and the loss of carbon and nutrients associated with traditional methods (Popa & Green, 2012).

## 1.1. LarWar Process

The Larvae for Wastewater Treatment and Resource Recovery (LarWaR) process is an innovative biological treatment approach that utilizes Black Soldier Fly (BSF) larvae (*Hermetia illucens*) for high-organic-content wastewater treatment. This method integrates wastewater stabilization with biomass recovery, aligning with circular economy principles. A key challenge, larval mortality in liquid environments, is addressed through a patented solution employing a porous support medium, facilitating larval mobility and survival (Grossule et al., 2023).

This bio-based process is particularly effective for treating wastewater with high organic loads, such as municipal landfill leachate and food industry effluents. Additionally, it enables resource recovery by converting organic waste into larval biomass, which can be repurposed for animal feed or biofuel production (Grossule et al., 2023).

### 1.1.1 Key Features of the LarWar Process

#### *1.1.1.1. Technical Innovation*

BSFL are terrestrial organisms that typically cannot survive in liquid environments, but to address this issue, a porous material is introduced into the reactors, which allows larvae to dive into the liquid to feed and breathe. This method was patented by Grossule and Cossu, 2021 (IT patent 102021000016700), paving the way for practical application in wastewater treatment.

#### *1.1.1.2. Waste Treatment and Resource Recovery*

The BSFL process couples the wastewater treatment process with the production of valuable larval biomass, which can be used for;

- Animal feed
- Biofuel production
- Other high-value products (fatty acids for cosmetics, pharmaceuticals, and chemicals) (Grossule et al., 2023)

This process helps to follow Circular Economy Principles and the European Green Deal by minimizing waste and maximizing the recovery of resources.

#### *1.1.1.3. Environmental and Economic Advantages*

The LarWaR process achieves high treatment efficiency, removing up to 95% of Total Organic Carbon (TOC) and Chemical Oxygen Demand (COD). It also has shorter Hydraulic Retention Times (HRT) than conventional systems, making it a faster treatment process. Additionally:

- It produces less sludge compared to conventional methods.

- It is more cost-effective and environmentally friendly, minimizing energy and aeration requirements.
- BSFL systems effectively handle seasonal organic load variations, such as peak production periods in industries like wineries.

### 1.1.2. Advantages of LarWar Over Conventional Processes

#### *1.1.2.1. Higher Removal Kinetics:*

- LarWaR achieves removal rates 3x faster than conventional aerobic methods, reducing treatment times and reactor sizes.

#### *1.1.2.2. Resource Recovery:*

- Converts HOC wastewater into biomass and other valuable resources, unlike traditional systems that produce excess sludge.

#### *1.1.2.3. Cost-Effectiveness:*

- Minimal aeration requirements reduce energy costs.
- Lower sludge generation decreases disposal costs.

#### *1.1.2.4. Sustainability:*

- The circular approach avoids downcycling, maximizing the recovery of high-value materials.

### 1.1.3. Process Performance

By integrating advanced bioconversion and sustainable treatment practices, the LarWaR process has demonstrated significant potential as an eco-friendly alternative to conventional wastewater treatment methods. It offers a promising pathway for the sustainable management of high-organic waste streams, transforming them into valuable resources while minimizing environmental impacts.

The LarWaR process excels in treating wastewater with high organic content (HOC). Unlike the simpler microorganisms used in conventional treatments, Black Soldier Fly Larvae (BSFL) are more complex and rely on two fundamental conditions to achieve optimal performance. These are organic content threshold, diet quality, and macronutrient balance. In the organic content threshold, BSFL requires sufficiently high concentrations of organic material in wastewater to sustain their growth and metabolism. Below a critical Treatment Threshold Limit (TTL), larvae performance declines sharply. About Diet Quality and Macronutrient Balance, the success of BSFL treatment depends heavily on providing a well-balanced macronutrient diet, particularly adequate protein and carbohydrate content. An imbalanced diet can limit larval growth and substrate conversion (Grossule et al., 2021).

## 1.2. Life Cycle of BSFL

The rapid growth in solid waste generation, driven by population increase, urbanization, economic growth, and changing consumer behaviors, is placing significant strain on current waste management systems. Estimates show that global solid waste production will surge from 2 billion tons in 2016 to 3.4 billion tons by 2050, emphasizing the need for sustainable and effective treatment methods. This rising waste volume contributes to the contamination of water, air, and soil, posing serious environmental challenges.

Black Soldier Fly Larvae (BSFL) bioconversion offers an innovative and efficient alternative to address these issues. BSFL can significantly reduce waste volume while transforming it into valuable by-products like high-protein animal feed and organic fertilizers. Additionally, BSFL systems provide integrated benefits by tackling not only organic solid waste but also potentially managing organic sludge and wastewater residues. This aligns with circular economy principles, where waste is repurposed into valuable resources.

Considering the surging waste crisis and the shortcomings of traditional methods, BSFL bioconversion stands out as a promising solution. Its ability to process organic waste efficiently while producing resource-rich outputs makes it an economical, sustainable, and environmentally friendly approach to managing waste and recovering valuable resources globally (Amrul et al., 2022).

*Figure 1. Visual Representation of BSL Larvae*

The black soldier fly (BSF), *Hermetia illucens*, is a true fly (Diptera) from the Stratiomyidae family, with significant potential as a cost-effective solution for recycling biological waste. Black Soldier Fly Larvae (BSFL) are efficient at converting waste into valuable biomass, which contains over 40% protein and 30% fat. These larvae, which can be seen in Figure 1 can reduce compost and manure while serving as a sustainable, reliable feed source. Their short life cycle allows for large-scale, continuous production, ensuring a reliable food source. Due to their natural waste-reduction abilities, BSFL is ideal for mass production, offering a cost-effective and sustainable solution (Park, 2016). Unlike other fly species, Hermetia illucens is not attracted to human residences, reducing the risk of disease transmission (Newton, 2004). Originally found in the tropical, subtropical, and northern regions of the Americas, BSFs are now present in both tropical and temperate regions worldwide. BSF adults are weak fliers and typically rest on plants throughout the day, living for about two weeks, during which they only consume water. Unlike other flies, BSF adults lack biting and feeding mechanisms, as they do not have a stinger, mouthparts, or digestive organs. They measure 15–20 mm in length, with males having a bronze abdomen and females a reddish-brown one. The adult female mates and lays eggs only once in her lifetime. BSFs are eurygamous, meaning they require expansive areas for mating flights (Amrul et al., 2022).

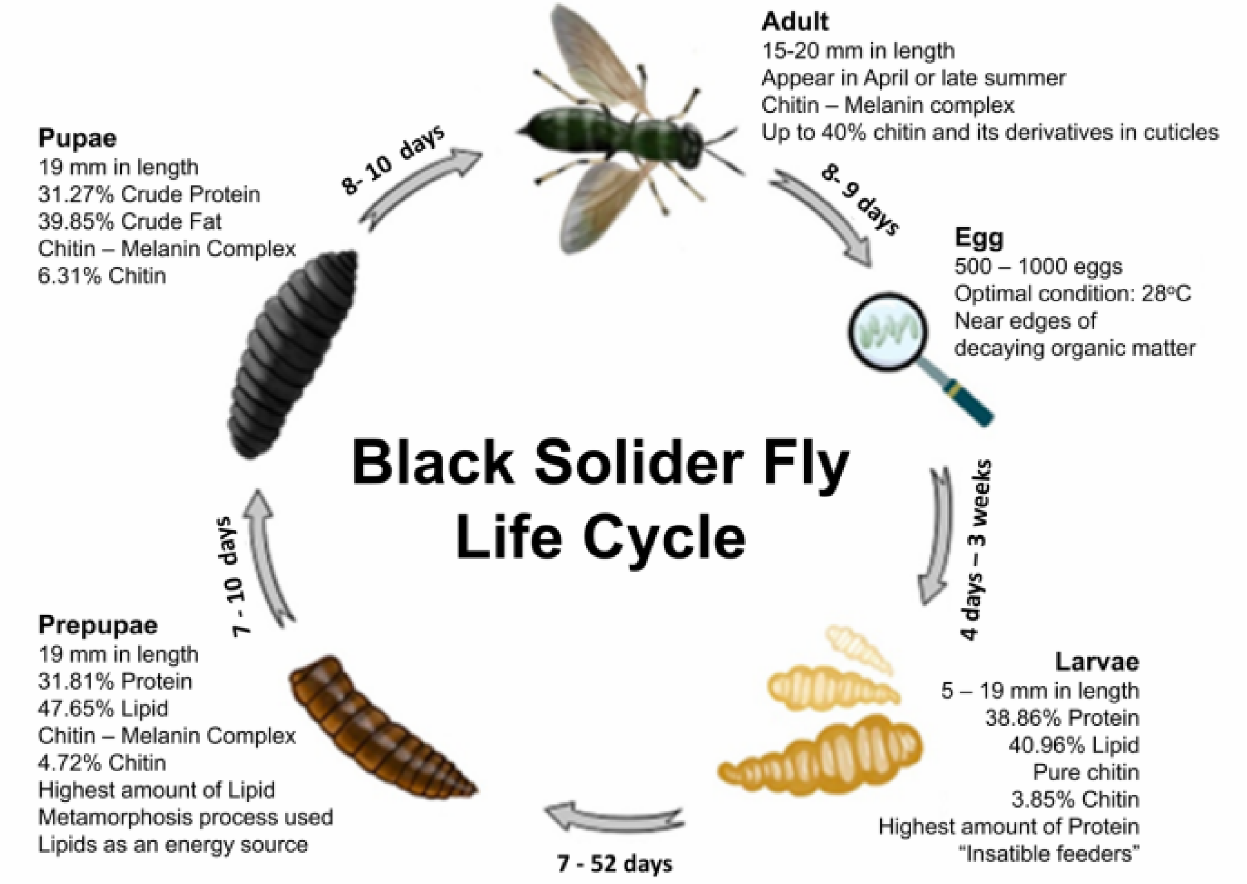

*Figure 2. Developmental Stages of BSF*

The life cycle of BLF can be divided into four stages, which are the egg stage, larval stage, pupal stage, and adult stage. These four stages can be seen in the Figure 2.

Egg Stage Duration: Around 4-5 days

Female Black Soldier Flies lay between 500 and 1000 eggs in clusters near decaying organic material, not directly inside it. The eggs are tiny (~1 mm), creamy white, and hatch into larvae in a few days.

1. Larval Stage Duration: Around 14-50 days (can extend to up to 6 months in poor condition)

Once hatched, the larvae begin feeding on organic waste immediately. They are creamy white, reaching around 27 mm in length and 6 mm in width when fully grown, with a small black head. This stage is vital as the larvae are highly efficient at breaking down organic matter like food scraps and manure while accumulating nutrients (over 40% protein and 30% fat) to sustain further development. In ideal conditions (27-30°C, around 70% humidity), they grow to their full size.

2. Pupal Stage Duration: Around 2 weeks

   At the final larval molt (sixth instar), the larvae stop feeding and seek a dry, dark place to pupate. The outer exoskeleton hardens and darkens as the larvae transform into pupae inside the protective case.

3. Adult Stage Duration: About 8-10 days

   Adult Black Soldier Flies measure about 2 cm in length and have black bodies and long antennae. They lack functional mouthparts and cannot eat, surviving on fat reserves from their larval stage and drinking water droplets. Mating occurs in flight, and after mating, females lay eggs and die, completing the life cycle (Müller et al., 2017).

Life Cycle Summary

Under optimal conditions (27°C and suitable humidity), the complete life cycle from egg to adult lasts about 6-8 weeks:

- Egg stage: 4-5 days
- Larval stage: 14-50 days
- Pupal stage: ~2 weeks
- Adult stage: 8-10 days

Black Soldier Fly (BSF) larvae are known for their adaptability to various environmental conditions, food shortages, oxygen deficiencies, resistance to insecticides, and competition with other insects. However, environmental factors, as well as the quality and quantity of food, can significantly influence their development. Key factors affecting BSF growth include food quantity, moisture, temperature, photo period, and pH levels (Grossule & Lavagnolo, 2019).

Several studies, including Tomberlin et al. (2002), suggest that humidity levels between 30-80% are ideal for BSF larvae, with humidity enhancing developmental success. Although BSF larvae can grow in liquid environments, they prefer moist or semi-solid conditions. BSF larvae can feed on a broad range of decomposing organic matter, such as kitchen waste, animal manure, spoiled food, fish offal, and even human waste.

The research by Popa and Green (2012) explored using BSF larvae for leachate treatment and found they could reduce chemical oxygen demand (COD) and volatile fatty acids (VFAs) while growing on organic leachate from fermented food scraps and plant material. The larvae's preferred food intake was 1 mL of organic leachate per larvae per week.

In summary, the larvae thrive under controlled conditions involving balanced food, optimal moisture (65–80%), temperature (around 27°C), and a suitable photoperiod. Additionally, the larvae are processed effectively when they are 5–7 days old.

## 1.3. The BSFL Optimum Nutrient Diet

The Black Soldier Fly (BSF) demonstrates a remarkable ability to adapt to various environmental conditions, including food scarcity, oxygen deficiencies, and exposure to insecticides and pesticides (Grossule & Lavagnolo, 2019). The biomass of BSF larvae (BSFL) is composed of 32-58% protein and 15-39% lipids (dry weight), making it a valuable resource for animal feed production (Gold et al., 2018). Nevertheless, the development rate and overall success of BSF larvae can be significantly influenced by factors such as environmental conditions and the quality or availability of food (Grossule & Lavagnolo, 2019).

### 1.3.1. Proteins

Proteins are terms of great influence with regard to Black Soldier Fly Larvae; they directly fuel the growth of the organism, its development, and overall biomass productivity. Larvae need protein for tissue synthesis, enzyme formation, and a myriad of other cellular components vital for efficient metabolic processes. Black Soldier Fly Larvae (BSFL) to communicate directly: up to 37% of protein contributes to growth rate enhancement and efficiency of feed conversion to the prepupal stage. For larval growth, biomass production, and maintenance of high metabolic activity, diets should be high protein and provide a balanced composition of amino acids ranging from 23% to 37% for tissue development. Besides, other protein-rich substrates like brewers' spent grain and wheat bran are too much to support these functions. However, excess protein beyond 37% can lead to the metabolic excretion leading to toxic byproducts like ammonia and uric acid. This can ultimately result in malnutrition, increased mortality, reduced feed efficiency, and lower output (Grossule et al., 2020). Diets that are too protein-rich may also result in inefficient substrate conversion and have increased mortality rates, further reducing overall larval performance (Grossule et al., 2024). Moderate protein content contributes to optimal larval development without risking excess nitrogen byproducts.

Role of Proteins:

- Tissue Development: Proteins provide amino acids that are crucial for the development of larval muscles and tissues.
- Growth Rates: Diets rich in proteins significantly reduce development times, enabling faster maturation.
- Biomass Efficiency: Higher protein content in the diet improves feed conversion efficiency and results in greater protein content within larval biomass, making it ideal for use in animal feed production.

Optimal Protein Content promotes:

- Faster larval development, reducing the time to reach prepupal stages.
- Greater final larval weight and biomass yield.
- Enhanced nutrient recycling efficiency during biowaste treatment.

Sources: Protein-rich substrates like brewers' spent grain and wheat bran offer balanced amino acids for better survival and development (Grossule et al., 2020).

### 1.3.2. Carbohydrates

Proteins play a pivotal role in Black Soldier Fly Larvae; they coordinate growth for the insects, including development and overall biomass productivity. Protein is necessary for tissue synthesis, enzyme formation, and a wide range of cellular components, all of which are critical for optimal metabolic processes. Further allude exactly to Black Soldier Fly Larvae (BSFL): essentially, up to 37% protein contributes towards growth rate acceleration and substrate conversion efficiency to the prepupa state. To sustain high metabolic activities, the preferred diets for larval growth, biomass production, and general maintenance must be high in protein. This involves delivering well-balanced amino acid compositions ranging from 23% to 37% toward tissue development. Finally, other alternative protein sources, such as brewers' spent grains and wheat bran, are too much to support these functions. However, excess protein beyond 37% can lead to metabolic excretion, leading to toxic byproducts like ammonia and uric acid. The excess nitrogen byproducts would ultimately render larvae malnourished, increasing mortality rates, reducing feed efficiency, and reducing output (Grossule et al., 2020). Diets that are too protein-rich may also result in inefficient substrate conversion and significantly increased mortality, all of which reduce overall larval performance (Grossule et al., 2024). Moderate protein content leads to optimal larval development without the risk of excess nitrogenous byproducts.

Role of Carbohydrates:

Energy Supply: Carbohydrates are metabolized for energy, which powers digestion, nutrient assimilation, and movement.

- Nutrient Conversion: When balanced with protein, carbohydrates enhance nutrient utilization efficiency without impairing development time.
- Lipid Accumulation: Excess carbohydrates result in fat storage, increasing larval lipid content at the cost of slower development.

Protein-to-Carbohydrate Ratios:

The ideal protein-to-carbohydrate balance ensures efficient energy utilization and growth:

- Balanced or Protein-Biased Ratios (1:1 to 4:1):

    - Promote faster growth rates, higher biomass production, and efficient development times.
- Carbohydrate-Biased Ratios (1:2 to 1:4):
    - This leads to increased lipid accumulation in larvae but results in prolonged development times and lower overall conversion efficiency.

### 1.3.3. Lipids

Along with proteins and carbs, lipids are an essential dietary component that offers a concentrated source of energy. However, to prevent negative effects on growth rates and digestion it is imperative to maintain an appropriate lipid content. A vital source of energy for Black Soldier Fly Larvae (BSFL) lipids are a broad class of organic compounds that include fats and oils. They are also vital for long-term energy storage. The larvae need to carefully control their intake of lipids even though they act as a secondary energy source and aid in the accumulation of fat reserves for survival particularly during the pupal and adult stages. After reaching adulthood and ceasing to feed adult flies depend on these fat reserves highlighting the fact that lipids offer a concentrated source of energy and are an essential dietary component in addition to proteins and carbohydrates. To prevent negative impacts on growth rates and digestion it is crucial to maintain an adequate lipid content. Black soldier fly larvae (BSFL) rely heavily on lipids a broad class of organic compounds that include fats and oils as an energy source and for long-term energy storage. The larvae need to carefully control their intake of lipids even though they act as a secondary energy source and aid in the accumulation of fat reserves for survival particularly during the pupal and adult stages. The necessity of larvae building up adequate fat reserves is highlighted by the fact that adult flies depend on these fat stores once they reach maturity and cease feeding (Grossule et al. (2024). However, the amount of fat in their diet needs to be managed because too much fat can hinder growth and survival which lowers waste conversion efficiency and larval performance. According to research the amount of lipids in an artificial diet shouldn't be more than 30 to 35 percent because this can make it more difficult for the larvae to efficiently turn waste into biomass (Grossule et al. in 2023). In contrast, larvae that consume a moderate amount of fat particularly when paired with a higher carbohydrate content are able to maintain efficient growth and biomass production while also building up sufficient fat reserves. In order to maximize the overall efficacy of the waste treatment process and guarantee larval performance, lipids should be carefully balanced even though they are essential for energy storage and possible biodiesel production. Of larvae building up adequate fat stores (Grossule et al. 2024). However, the amount of fat in their diet needs to be managed because too much fat can hinder growth and survival which lowers waste conversion efficiency and larval performance. An artificial diet's lipid content should not be greater than 30 to 35 percent according to research since this can make it more difficult for the larvae to efficiently turn waste into biomass (Grossule et al., 2023). A moderate lipid intake on the other hand particularly when paired with a higher carbohydrate content aids in the development of sufficient fat reserves while preserving effective growth and biomass production in larvae. Lipids must, therefore be carefully balanced to maximize the overall efficacy of the waste treatment process and guarantee larval performance even though they are essential for energy storage and possible biodiesel production.

Role of Lipids:

- Energy Storage: Lipids act as long-term energy reserves, supporting metabolic demands during larval development.
- Nutrient Density: Moderate lipid levels enhance overall biomass nutritional quality, making larvae valuable as an energy-dense feedstock or raw material for biofuel production.

Optimal Lipid Content promotes:

- Supplement carbohydrates for energy metabolism.
- Enhance larval fat accumulation for biofuel applications without impairing protein development.

Excess Lipid Effects:

Diets excessively rich in lipids (e.g., food residues with high oil/fat content) can:

- Slow development rates, as larvae struggle to process excess fats.
- Decrease overall bioconversion efficiency during waste treatment.

Sources: Food waste, vegetable, and fruit waste provide a balanced lipid content suitable for larval development.

*Table 1. Optimal Macronutrients for BSFL*

| Parameter | Optimal Range | Effect on BSFL Performance |
|---|---|---|
| Protein | 23–37% | Promotes growth, faster development, and high biomass protein yield. |
| Carbohydrates | 40-60% | Energy supply supports growth efficiency |
| Lipids | up to 30-35% | Provides energy; supports fat accumulation without impairing growth. |

A well-balanced BSFL diet includes 23-37% proteins, up to 35% lipids, and 40-45% carbohydrates. These values and sources of macronutrients can be seen in Table 1. Achieving this balance maximizes larval growth, biomass production, and nutrient conversion efficiency while minimizing development time. Mixed biowaste streams, such as animal manure, brewery by-products, and organic kitchen waste, can provide an economically viable and nutritionally adequate diet for sustainable BSFL production.

### 1.3.4. Optimal Macronutrient Ratios

Maintaining a balanced macronutrient composition is essential for BSFL, as excessive or insufficient levels of proteins, carbohydrates, or lipids can adversely affect the survival and waste conversion efficiency of Black Soldier Fly larvae (BSFL). BSFL exhibits optimal performance when the diet is characterized by a higher proportion of non-fiber carbohydrates (NFC) compared to proteins (P) and lipids (L). An imbalance, particularly an excess of proteins or lipids, may result in reduced survival rates, slower growth, and suboptimal bioconversion efficiency. Therefore, optimizing the P:C:L ratio is necessary (Grossule et al., 2024).

Figure 3. Good quality substrates (GQS), achieving SR > 80% and RE > 40%, are identified using a triangular chart based on the relative proportions of proteins (X_P), non-fiber carbohydrates (X_NFC), and lipids (X_L) (Grossule et al., 2024)Figure 3 presents methods for identifying good-quality substrates (GQS) based on the relative proportions of proteins (X_P), non-fiber carbohydrates (X_NFC), and lipids (X_L). GQS are defined as substrates that achieve a survival rate (SR) > 80% and a reduction efficiency (RE) > 40%.

The proportions of $X_P$, $X_{NFC}$, and $X_L$ are calculated by dividing the concentration of each macronutrient (as a percentage of the substrate's dry weight) by the total macronutrient concentration (∑PCL) in the same substrate.

The ternary diagram in Figure 3 is a triangular representation of substrate composition. Each corner of the triangle represents 100% dominance of a single macronutrient:

- Bottom-left corner: 100% Proteins ($X_P$ = 1.0, $X_{NFC}$ = 0, $X_L$ = 0)
- Bottom-right corner: 100% Non-fiber Carbohydrates ($X_{NFC}$ = 1.0, $X_P$ = 0, $X_L$ = 0)
- Top corner: 100% Lipids ($X_L$ = 1.0, $X_P$ = 0, $X_{NFC}$ = 0)

Each point inside the triangle represents a different ratio of proteins, carbohydrates, and lipids. The green-shaded region within the diagram identifies substrates that achieve optimal process performance, specifically:

- Survival Rate (SR) > 80%
- Reduction Efficiency (RE) > 40%

Substrates outside the green area do not meet these performance thresholds due to imbalanced macronutrient composition.

A substrate qualifies as a Good Quality Substrate (GQS) when it meets the following conditions:

1. Lipids ($X_L$) must be below 60%

    - Condition: $X_L < 0.6$

    - Reason: Excessive lipid content negatively impacts BSFL growth and reduces substrate digestibility.

2. Protein ($X_P$) must be above 5%
    - Condition: $X_P > 0.05$
    - Reason: BSFL requires a minimum protein level for optimal survival and development.
3. If protein content exceeds 50%, sufficient carbohydrates are required
    - Condition: $X_P > 0.5 \rightarrow X_{NFC} > 0.2$
    - Reason: High-protein diets need at least 20% carbohydrates to balance nitrogen metabolism and avoid toxic by-product accumulation (e.g., ammonia).
4. If protein content is between 5% and 15%, carbohydrates must exceed lipids
    - Condition: $0.05 \leq X_P \leq 0.15 \rightarrow X_{NFC} > X_L$
    - Reason: When protein levels are low to moderate, having more carbohydrates than lipids ensure efficient waste reduction and larval survival.

- A well-balanced substrate contains:
    - Higher carbohydrate content ($X_{NFC} > X_P$ and $X_L$)
    - Moderate protein levels ($X_P$ between 0.1–0.5)
    - Controlled lipid content ($X_L < 0.6$) (Grossule et al., 2024).

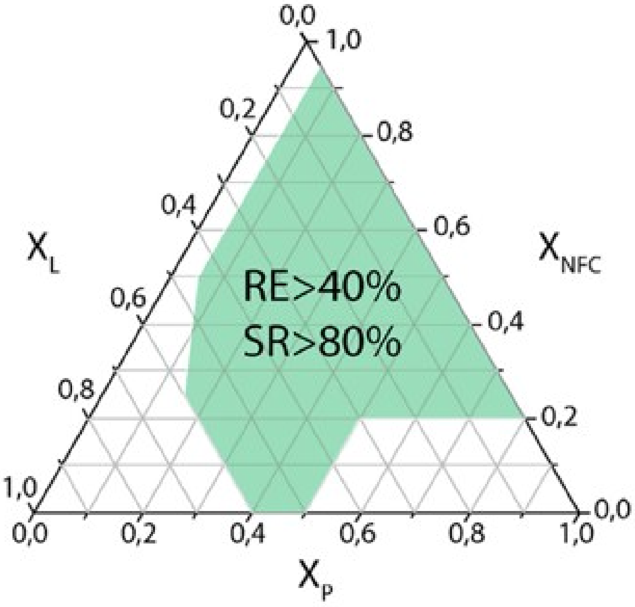

*Figure 3. Good quality substrates (GQS), achieving SR > 80% and RE > 40%, are identified using a triangular chart based on the relative proportions of proteins (X_P), non-fiber carbohydrates (X_NFC), and lipids (X_L) (Grossule et al., 2024)*

## 1.4. Research Objective

The main objective of this research is to determine the threshold organic concentration at which Black Soldier Fly (BSF) larvae can effectively process wastewater, considering the influence of varying organic loads and diet composition on their growth and substrate degradation efficiency. This study aims to quantify the larvae's capacity to convert organic matter into biomass under different Total Organic Carbon (TOC) conditions, assessing key parameters such as substrate removal efficiency, mortality rates, and biomass yield. Unlike previous studies, this research investigates a lower minimum TOC concentration to evaluate larval performance in wastewater treatment systems with balanced macronutrient availability. By systematically monitoring variations in larval wet weight, prepupation rates, and TOC reduction, this study seeks to establish the minimum organic concentration required for effective BSF-based wastewater treatment, contributing to the optimization and scalability of this approach for sustainable water management.

## 1.5. Overview of Prior Tests on Concentrations and Organic Loads

Numerous studies have assessed the capacity of Black Soldier Fly larvae (BSFL) to treat diverse substrates, such as artificial wastewater and leachates, using varying concentrations and organic loads on *Table 2*.These investigations primarily focused on understanding how substrate concentration and organic load influence larval growth, substrate consumption, and nutrient

### 1.5.1. Summary of Data from Literature

Table 2 consolidates data from multiple studies, categorizing outcomes based on substrate concentration, organic load, larval growth, and mortality rates. Key findings include:

- High substrate concentrations (≥1,566 mg/L) were generally associated with improved larval growth and lower mortality rates, indicating effective waste conversion.
- Lower concentrations and organic loads often resulted in reduced final wet weight and increased mortality, suggesting the presence of critical thresholds necessary for BSFL viability.
- Extremely high organic loads (≥5 mg/larva/day) showed negative effects, leading to increased mortality rates, as seen in studies where values exceeded this threshold.
- Artificial leachate at moderate concentrations (1,952 to 6,940 mg/L) demonstrated progressive improvements in larval wet weight with increasing organic loads, with optimal results observed around 6,940 mg/L and 2.6 mg/larva/day.
- Variations in substrate type influenced results: organic-rich wastewater such as bakery wastewater supported higher larval growth (final weight: 109 mg) compared to slaughterhouse or juice production wastewater, which resulted in lower weight and higher mortality.
- Green-labeled results in Table 2 represent successful tests, typically linked to substrate concentrations above 1,500 mg/L and organic loads within a moderate range (0.5 to 2.6 mg/larva/day).

In conclusion, while BSFL demonstrates adaptability under various conditions, the success of waste treatment largely depends on maintaining sufficient TOC concentrations and organic loads. This understanding informs future experimental designs aimed at determining the minimum effective thresholds to ensure larval viability and waste conversion efficiency.

*Table 2. Summary of Concentrations and Organic Loads Investigated in Previous Studies*

| **Substrate** | **Concentration (mg/L)** | **Carbon Load (mg/larva/d)** | **Final wet weight (mg)** | **Mortality (%)** | **Reference** |
|---|---|---|---|---|---|
| Artificial leachate | 1.952,00 | 0,73 | 38,3 | 7 | **Grossule et al 2023: Effect of organic load and concentrations** |
| | 1.952,00 | 0,37 | 37,5 | 5 | |
| | 1.952,00 | 0,18 | 36,9 | 4 | |
| | 1.952,00 | 0,09 | 35 | 2,5 | |
| | 3.650,00 | 1,37 | 46 | 6 | |
| | 3.650,00 | 0,68 | 42,5 | 4 | |
| | 3.650,00 | 0,34 | 40,2 | 3 | |
| | 3.650,00 | 0,17 | 38,7 | 2,5 | |
| | 6.940,00 | 2,6 | 67,9 | 0 | |
| | 6.940,00 | 1,3 | 64,2 | 0 | |
| | 6.940,00 | 0,65 | 58,3 | 1 | |
| | 6.940,00 | 0,33 | 52,6 | 3 | |
| Artificial leachate | 3.494,00 | 3,74 | 48 | 20 | **Grossule et al 2022: Preparation of artificial MSW leachate for treatment studies** |
| | 2.932,00 | 3,14 | 60 | 15 | |
| | 5.510,00 | 5,9 | 53 | 31 | |
| Artificial leachate | 3.827,00 | 4,1 | 48 | 32,55 | **Grossule et al. 2022.07: Different** |
| | 3.430,00 | 3,68 | 48 | 20 | |

|  |  |  |  |  |  |
|---|---|---|---|---|---|
|  | 2.960,00 | 3,17 | 55 | 15 | **degrees of biodegradability and oxidation of organic content** |
|  | 3.790,00 | 4,06 | 66 | 17,5 |  |
|  | 3.760,00 | 4,03 | 80 | 10 |  |
|  | 3.409,00 | 3,65 | 92 | 6 |  |
| Artificial WW (Valox) | 2.740,00 | 0,14 | 57,3 | 19,3 | **Possanzini** |
|  | 2.740,00 | 0,14 | 59,1 | 21,6 |  |
| Artificial WW (Kaldnes) | 2.740,00 | 0,14 | 50,1 | 20,3 |  |
|  | 2.740,00 | 0,14 | 50,2 | 14,1 |  |
|  | 2.740,00 | 0,14 | 52,8 | 7,4 |  |
| Artificial WW (Geomat) | 2.740,00 | 0,14 | 56,8 | 30,9 |  |
|  | 2.740,00 | 0,14 | 59,7 | 29,8 |  |
|  | 2.740,00 | 0,14 | 59,1 | 25,5 |  |
| Bakery WW | 1.902,00 | 0,51 | 109 | 17 | **Feratović** |
| Brewery WW | 1.566,00 | 0,42 | 53 | 15 |  |
| Diary WW | 358 | 0,1 | 26,3 | 10 |  |
| Juice production WW | 744 | 0,2 | 27,6 | 65 |  |
| Slaughterhouse WW | 433 | 0,12 | 17 | 43 |  |
| Winery WW | 1.842,00 | 0,49 | 61,2 | 10 |  |

## 2. Materials and Methods

### 2.1. Research Program

The experiment was conducted to identify the optimal range of carbon load and total organic carbon (TOC) concentration that would promote the growth of *Hermetia illucens* larvae and enhance the efficiency of organic substrate conversion in continuous reactors. The primary goal was to determine the minimum and maximum conditions that support optimal larval development and maximize substrate degradation.

The larvae used in the study were supported by Kaldnes®. The material was saturated with a solution of proteins and glucose dissolved in distilled water, simulating artificial wastewater conditions.

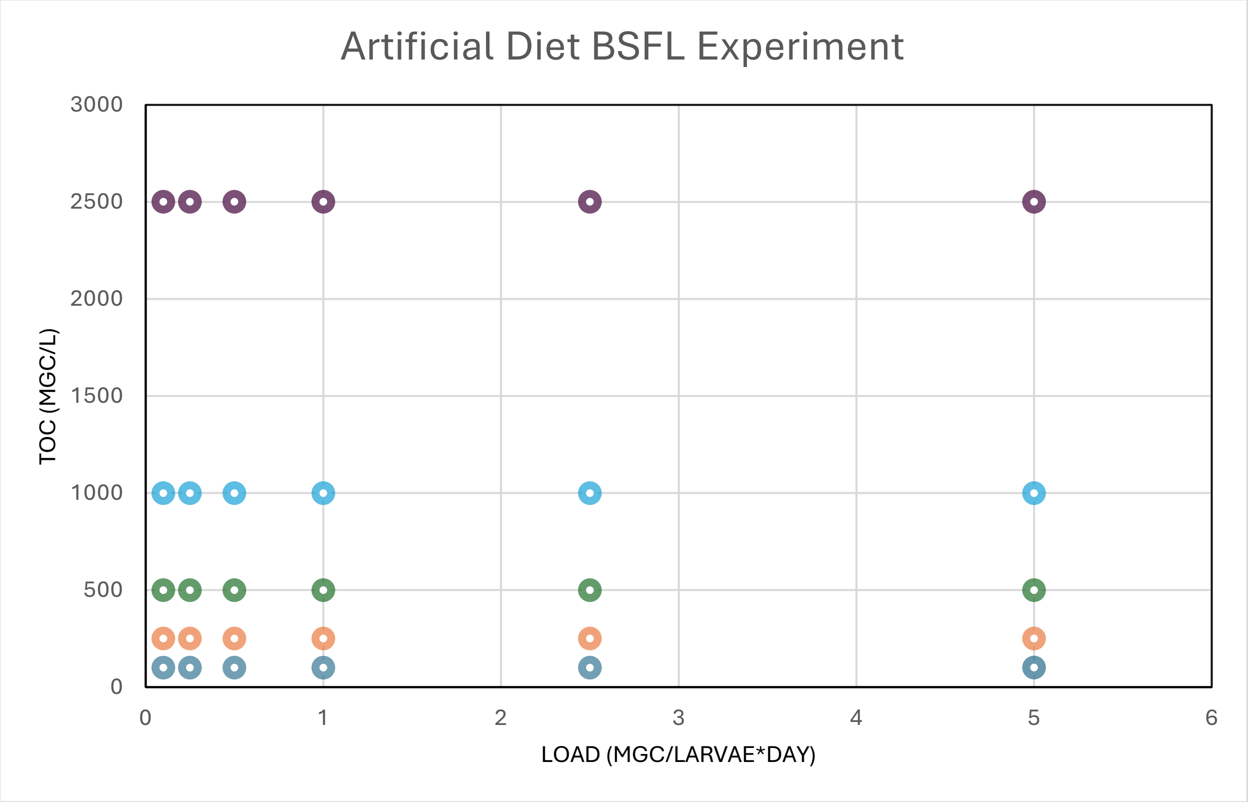

*Figure 4. Artificial Diet of BSFL Experiment*

Six carbon load levels were tested: 0.1, 0.25, 0.5, 1, 2.5, and 5 mgC/larva, with initial TOC concentrations of 100 mgC/L, 250 mgC/L, 500 mgC/L, 1000 mgC/L and 2500 mgC/L can be seen in Figure 4. These values were selected to investigate the boundaries of larval performance and substrate conversion efficiency. Artificial wastewater was prepared by dissolving proteins and glucose in distilled water to create a nutrient-rich medium, analogous to organic wastewater.

Larval growth and substrate consumption were closely monitored to identify the conditions ensuring effective larval development and high substrate conversion efficiency.

## 2.2. Experimental Setup

The experimental setup employed continuous reactors and batch reactors to evaluate the effects of different carbon loads and TOC concentrations on the growth and waste conversion efficiency of *Hermetia illucens* larvae. Each reactor was designed to maintain stable environmental conditions, using Kaldnes® as the inert support material due to its proven stability and effectiveness in supporting larval development. The continuous reactors are used for low organic loads and low TOC concentrations whereas batch reactors are used for higher organic loads and higher TOC concentrations.

The reactors, which consisted of plastic boxes, were prepared by adding junctions and holes to accommodate the necessary piping for feeding and waste collection. Each reactor was equipped with permeable non-woven fabric covering to prevent oviposition by other flies while ensuring proper air exchange. Additionally, a perforated plastic lid was used to promote air recirculation, creating an optimal balance between minimizing external interference and providing adequate oxygen for the larvae.

Before initiating the experiment, the first step was testing the pumps. The revolutions and timing of the pumps were carefully assessed, leading to the selection of five pumps, three large pumps, and two smaller ones. Based on the results obtained from these initial tests, the experimental setup, detailed in **Error! Reference source not found.**, was structured. After analyzing the data from Table 3 and considering the trial process for the pumps, it was decided that the pumps would operate for 15 minutes every 2 hours, continuously working for 5 days, 24 hours a day. This setup was chosen to ensure the optimal functioning of the pumps during the trial period.

*Table 3. Experimental Data*

|  |  |  |  |  |  | Working 5 days, 24 h, 15 min every 2 hour |  |
|---|---|---|---|---|---|---|---|
| Sample name | TOC | No.larvae | Load (mgC/larvae*day) | Pump_tube | REV | L/min |  |
| C1_1 | 100 | 273 | 1 | B2_4mm | 10.0 | 200 | 2 |
| C1_2.5 | 100 | 287 | 2.5 | B1_4mm | 30.0 | 200 | 5 |
| C1_5 | 100 | 199 | 5 | B1_8mm | 30.0 | 200 | 10 |
| C2_1 | 250 | 302 | 1 | S3_1mm | 7.0 | 500 | 2 |
| C2_2.5 | 250 | 269 | 2.5 | B3_4mm | 10.0 | 500 | 5 |
| C2_5 | 250 | 200 | 5 | B2_8mm | 10.0 | 500 | 10 |
| C3_1 | 500 | 180 | 1 | S1_1mm | 1.5 | 1000 | 2 |
| C3_2.5 | 500 | 242 | 2.5 | S3_1mm | 7.0 | 1000 | 5 |
| C3_5 | 500 | 269 | 5 | B3_4mm | 10.0 | 1000 | 10 |

Additionally, the tubing for the pumps was selected based on the calculations and trial outcomes, with tube sizes of 1 mm, 4 mm, and 8 mm chosen for the experiment. The load, total organic carbon (TOC) concentrations, and the number of larvae were also carefully calculated and adjusted to meet the specific conditions of the experiment. These calculations took into account the necessary flow rates, pump durations, and other relevant factors to ensure that the larvae could be properly maintained and observed under the required organic load and TOC conditions.

Thus, the experimental conditions were carefully planned, and the setup involved pumps running for 15 minutes every 2 hours, over a period of 5 days, ensuring a stable and controlled environment for the larvae testing.

Large bottles were used to supply feed and collect output for analysis. As observed in the setup, Figure 5, there were nine bottles for feeding (in) and nine bottles for waste collection (out)

*Figure 5. Setup for Continuous Test*

Each day, a fresh diet is prepared for the larvae and not fed. Incoming feed bottles (called "in-bottles") are filled with a freshly prepared diet solution initially formulated as TOC5000 and then dispersed to achieve target compositions. The effluent ingested by the larvae is collected in out-flow bottles, also known as out-flow bottles. The organic carbon (TOC) is dissolved, and the applied organic load is indicated in total on the labels of each bottle. For example, a bottle labeled C1_2.5 OUT indicates an out-flow bottle grown with 2.5 mgC/larva organic fraction and 100 mgC/L TOC. To monitor changes in TOC over time, 50 mL samples were taken from the outlet bottle each day. At the end of the week, after thorough mixing of the daily samples, a regular weekly sample was made and subjected to TOC analysis. This management strategy allowed TOC measurements to provide a detailed depiction of the organic load reduction during the intermittent period and to assess treatment results more precisely. The experiment was produced in nine separate forms, where three TOC section level tests were presented as C1, C2, and C3, and three carbon loads were 1, 2.5, and 5 mgC/larva. Each disease housed a certain number of larvae, which were recorded to receive a guaranteed certificate for payments between treatments. Larvae were fed at the same time each day, and daily TOC samples were taken for analysis. Concentrated wastewater solutions were prepared by dissolving glucose and whey protein in distilled water and assigning specific amounts to each TOC concentration. Usually, the highest concentration (C3) was prepared, and the lower concentration (C2 and C1) was obtained by calculated dilution. Environmental conditions were monitored to ensure optimal larval growth and substrate conversion, following the methods suggested by Grossule et al. (2020). All options were kept in a room at a constant 27°C with a 12-hour interval to simulate natural light formations and support regular larval parts. The experiment lasted 42 days (6 weeks). Larval growth was monitored weekly on the same day using an analytical balance to apply larval weight and recorded for data programs. TOC samples were also analyzed weekly, and results were tabulated for further analysis.

### 2.3 Feeding

This study applies a precise feeding protocol and monitoring structure to increase the efficiency and crescent of the transformation in the substrate of the Black Soldier Fly (BSF). A nutrient-rich artificial diet and normal formula, using a combination of protein, glucose, and distilled water, provide a suitable source of nutrition and high quality under experimental conditions. This protective component is expensive in terms of natural latte protein, contains essential amino acids, and protects glucose as the first carbohydrate source to support the metabolism and energy production of larvae. By strictly controlling the nutritional composition, the study accurately evaluated the effectiveness of various organic total carbon concentrations (TOC) and increased the efficiency of organic matter and substrate degradation, which feeds the larvae well.

A freshly prepared artificial diet was introduced into the continuous reactors each day at the same hour to ensure that the larvae received an optimum nutrients daily. The preparation process is initiated by creating a TOC5000 concentrate solution and diluting it according to the specific nutritional requirements.

The concentrated diet was prepared using 11.6 g of glucose and 12.8 g of protein per 2L of solution, ensuring a standardized composition across all trials, corresponding TOC5000 solution and bottle can be seen in the Figure 6.

*Figure 6. TOC5000 Solution*

Each treatment group was given an organic load, and the volume of substrate in each compartment was changed based on the transfer pattern. Higher organic load animals were given larger feeding volumes, whereas lower load animals were divided into smaller portions. This precise volumetric adjustment ensured that both the larvae of each unit and the appropriate nutritional status according to the tested TOC zone were maintained. The volume of concentrate (TOC5000) and the volume of diluted water in mL can be seen in Table 4.

*Table 4. Volume allocation based on load and TOC concentrations*

| **Volume of Concentrated Solution (mL)** | **Volume of Distilled Water (mL)** |
|---|---|
| 76 | 3740 |
| 201 | 9843 |
| 279 | 13653 |
| 85 | 1607 |
| 188 | 3580 |
| 280 | 5312 |
| 50 | 454 |
| 169 | 1523 |
| 377 | 3391 |
| **Total Volume of TOC5000 Needed (mL)** | **1705 mL** |

To maintain experimental consistency, the substrate was replaced daily, and the collected effluent from each reactor was stored for further analysis. At the end of each week, a composite sample from the accumulated outflows was analyzed to determine Total Organic Carbon (TOC) levels. These weekly TOC assessments provided critical insights into the extent of organic matter breakdown by the larvae, allowing researchers to evaluate the efficiency of substrate consumption across varying TOC concentrations and organic loads.

## 2.4. Operation and Monitoring

To ensure comprehensive monitoring of larval development and substrate degradation, multiple key parameters were tracked throughout the experiment: larval growth monitoring, TOC, substrate removal and conversion efficiency, and mortality and developmental monitoring.

### 2.4.1. Larval Growth Monitoring:

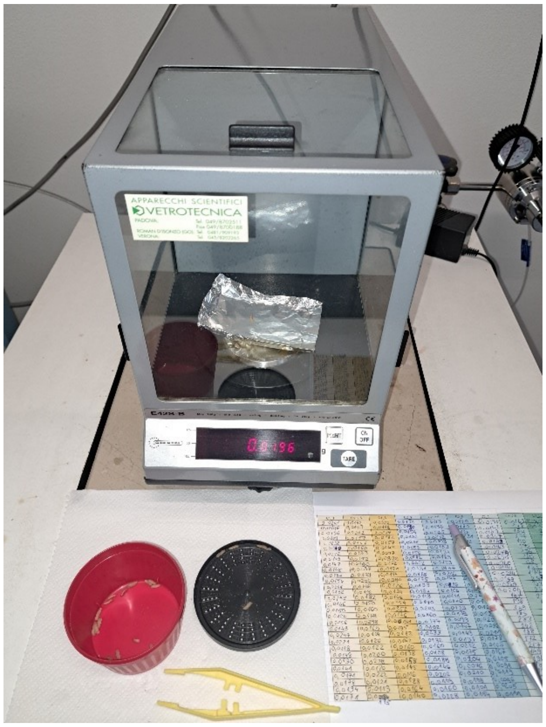

*Figure 7. Analytical Balance*

- Weekly measurements and records of the larvae's wet weight were made using a high-precision analytical balance to track their development and growth as well as for use in subsequent processing. It was easier to evaluate how well the larvae converted the given substrate into biomass, thanks to this measurement.
- •Every week at the same time and day, 30 larvae were chosen at random from each reactor, and their weights were recorded using an analytical balance (see Figure 7) to be processed.
- Every week, 270 larvae were chosen at random, and their weights were methodically noted for further examination.

### 2.4.2. Total Organic Carbon (TOC) Analysis:

Figure 8. Shimadzu TOC analyzer

- Weekly TOC measurements were performed on the collected effluent samples using a Shimadzu TOC analyzer, as can be seen in Figure 8, to quantify the efficiency of organic matter degradation. Each day, at the same time, a 50 mL sample was collected from the outlet bottles. At the end of each week, the daily samples were combined, and the total organic carbon (TOC) was measured to assess the overall degradation efficiency

### 2.4.3. Substrate Removal and Conversion Efficiency:

- The rate of substrate consumption and conversion efficiency were evaluated through substrate removal efficiency (%) and the calculation of yield (Y), representing the amount of larval biomass produced per unit of organic matter consumed.

### 2.4.4. Mortality and Developmental Monitoring:

- The mortality rate (percentage of larvae that did not survive) was recorded to assess how different TOC concentrations and organic loads affected larval survival.

The prepupation rate (percentage of larvae reaching the prepupal stage) was also monitored as a developmental indicator.

This comprehensive feeding, operation, and monitoring approach provided valuable data on substrate degradation dynamics, larval metabolism, and overall system performance. By systematically adjusting feeding volumes, tracking TOC levels, and monitoring larval growth, this study aimed to establish a detailed understanding of the relationship between organic load variations and substrate conversion efficiency in Black Soldier Fly larvae bioconversion systems.

## 2.5. Analytical Procedure

All relevant data were recorded during the study to ensure systematic observation and accurate experimental evaluation. Several important parameters, including yield, mortality, reduction efficiency, and specific substrate consumption rate, were determined using analytical formulas. These measurements provided vital details regarding the organic matter degrading capacity of larvae. Under different experimental conditions, the study investigated the relationships between total organic carbon (TOC) concentration and organic load to evaluate the efficiency of substrate conversion and larval development. This comprehensive approach allowed for a better understanding of how variations in organic load affect larval growth survival and overall system performance.

### 2.5.1. Yield :

$$Y = \frac{\Delta X}{\Delta S} \quad (1)$$

The yield (Y) ( eq1) represents the efficiency of larvae in converting organic matter into biomass. It is defined as the ratio of the change in larval biomass $\Delta X$to the change in substrate concentration $\Delta S$.

- $\Delta X$ (Change in larval biomass)

- $\Delta S$ (Change in substrate concentration)
- $Y$ (Yield): Dimensionless ratio

A higher yield indicates that the larvae effectively utilize organic material for biomass production. This parameter is essential for evaluating the efficiency of organic matter conversion in bioconversion processes.

2.5.2. Mortality:

$$\frac{number\ of\ dead\ larvae}{number\ of\ initial\ larvae} * 100 \tag{2}$$

The mortality rate quantifies the proportion of larvae that did not survive during the experimental period. It is calculated as the percentage of dead larvae relative to the initial larval population.

- Number of dead larvae: Unitless count
- Number of initial larvae: Unitless count
- Mortality rate: %

This parameter provides insight into the survival rate of the larvae under experimental conditions. A high mortality rate may indicate unfavorable environmental conditions, such as excessive organic loading, nutrient imbalances, or toxic effects of the substrate.

2.5.4. Reduction Efficiency:

$$\frac{S_{in} - S_{out}}{S_{in}} * 100 \tag{3}$$

Substrate reduction efficiency assesses the effectiveness of organic matter degradation by the larvae. It is defined as the percentage of organic matter removed from the wastewater during the treatment period.

- $S_{in}$ (Initial substrate concentration): mg TOC/L

- $S_{out}$ (Final substrate concentration after treatment): mg TOC/L
- Reduction efficiency: %

This parameter is crucial for determining the treatment performance of the larvae. A higher reduction efficiency indicates greater removal of organic matter, signifying effective biodegradation.

### 2.5.3. Specific Substrate Consumption Rate:

$$V_S = \frac{dS}{X_0 * dt} = \frac{(S_0 - S)}{X_0 * (t - t_0)} \tag{4}$$

The specific substrate consumption rate ($V_S$) measures the rate at which each larva consumes organic material per unit time. It is expressed as the amount of substrate removed per larvae per day. The specific substrate consumption rate ($V_S$), expressed in mgC/larva/day (Grossule et al., 2022), was determined on a weekly basis using TOC measurements and calculated according to the equation (4)

- $(S_0 - S)$ (Substrate removed): mg TOC
- $X_0$ (Initial number of larvae): Unitless count
- $(t - t_0)$ (Time interval): Days
- $V_S$ (Specific substrate consumption rate): mg TOC/larva/day

This parameter is essential for evaluating the metabolic activity of the larvae in organic matter degradation. A higher $V_S$ value indicates greater efficiency in substrate consumption, contributing to enhanced treatment performance.

## 3. Results and Discussion

This chapter presents the results of the experimental study, focusing on the key parameters used to evaluate the performance of Black Soldier Fly larvae (BSFL) in wastewater treatment under varying organic load and TOC concentration conditions, as previously discussed. The analysis encompasses larval growth, mortality, prepupation, yield, specific substrate consumption rate, and overall process efficiency.

The findings are examined concerning one another to provide a comprehensive understanding of how different feeding conditions influence larval development, survival rates, and substrate conversion potential. Particular attention is given to identifying the optimal conditions for maximizing biomass production while ensuring effective organic matter degradation. The discussion also contextualizes the results within existing literature, highlighting both consistencies and deviations from previous research on BSFL-based waste bioconversion systems.

## 3.1. Larval Development

This section presents the results of larval growth over time under different experimental conditions. The data is analyzed using six distinct organic loads (0.1, 0.25, 0.5, 1, 2.5, and 5) and five TOC concentrations (C1, C2, C3, C4 and C5). The trends observed in the data provide insights into the impact of these parameters on larval biomass accumulation.

For the lowest load of 0.1 (Figure 9 A) The minimum weight increase was observed across all loads. The maximum weight increase is in concentration C5, which reached around 35mg on week 5, while the other concentrations stayed at low weights. After 5th week there is a decrease in weight for concentration C5. When the second load is considered again the best performing concentration is C5. It showed a similar trend with load 0.1.

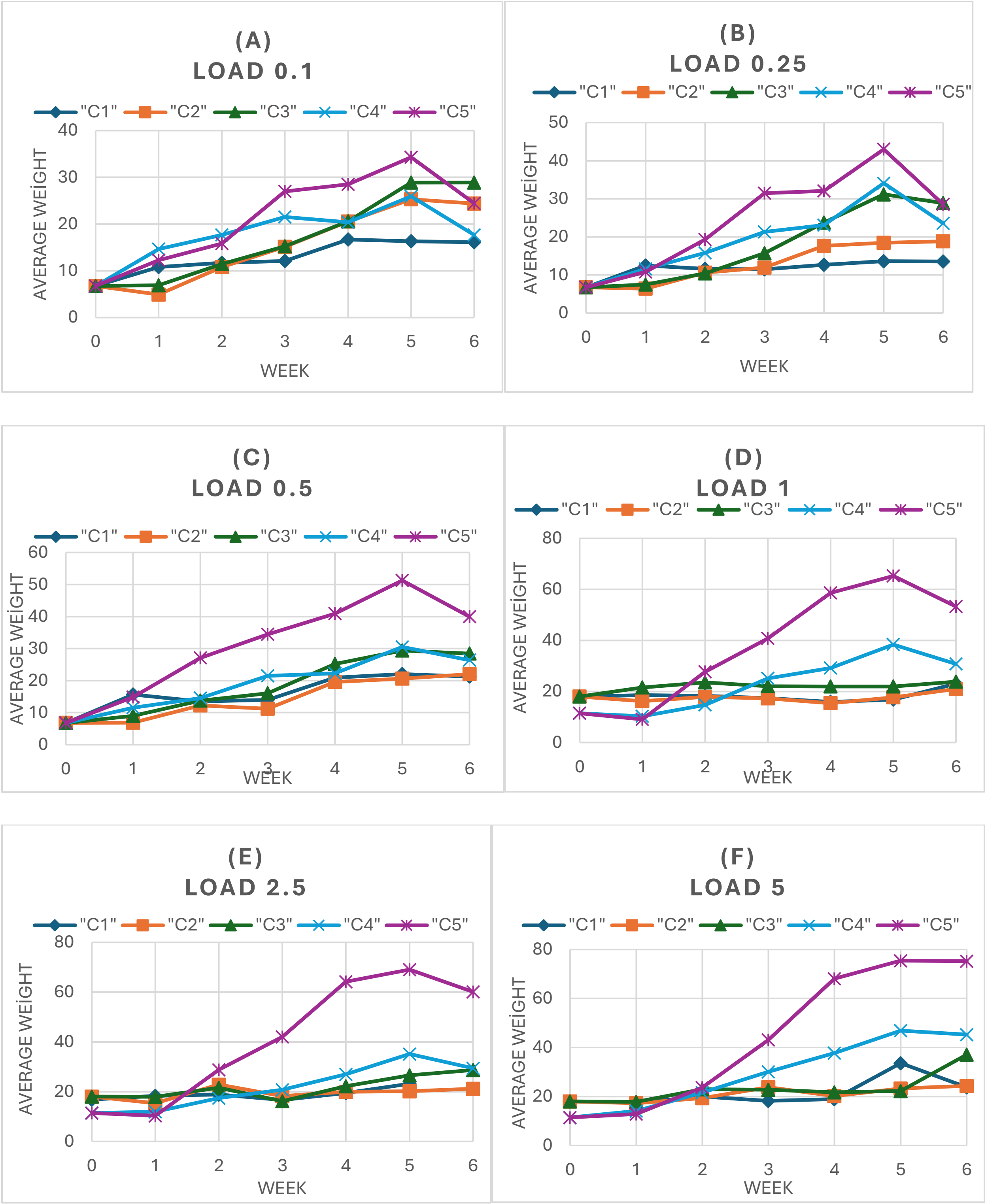

*Figure 9. BSF Larvae average weight over weeks for different loads: (A) load 0.1, (B) load 0.25, (C) load 0.5, (D) load 1, (E) load 2.5, and (F) load 5 across five different concentrations*

When it is considered the upcoming load of 0.5 in Figure 9, it becomes evident that C5 begins to exhibit superior performance compared to other concentrations, which remain at relatively low weights. This suggests that at lower organic loads, C5 already demonstrates a stronger growth response. As the organic load increases to 1, 2.5, and 5, the trend becomes even more pronounced, with C5 showing a significantly higher performance across all these conditions. In particular, both C5 and C4 outperform the lower concentrations at these increased loads; however, C4 remains below the performance level of C5. Notably, in Figure 9 (F), which represents the highest load of 5, C5 reaches a final weight of nearly 80 mg, despite starting the experiment with an initial weight of less than 20 mg. This substantial increase in weight highlights that under these specific conditions, C5 concentration, and a load of 5, the larvae exhibited remarkable growth and adaptation, indicating an optimal combination for enhanced biomass development.

### 3.2. Mortality

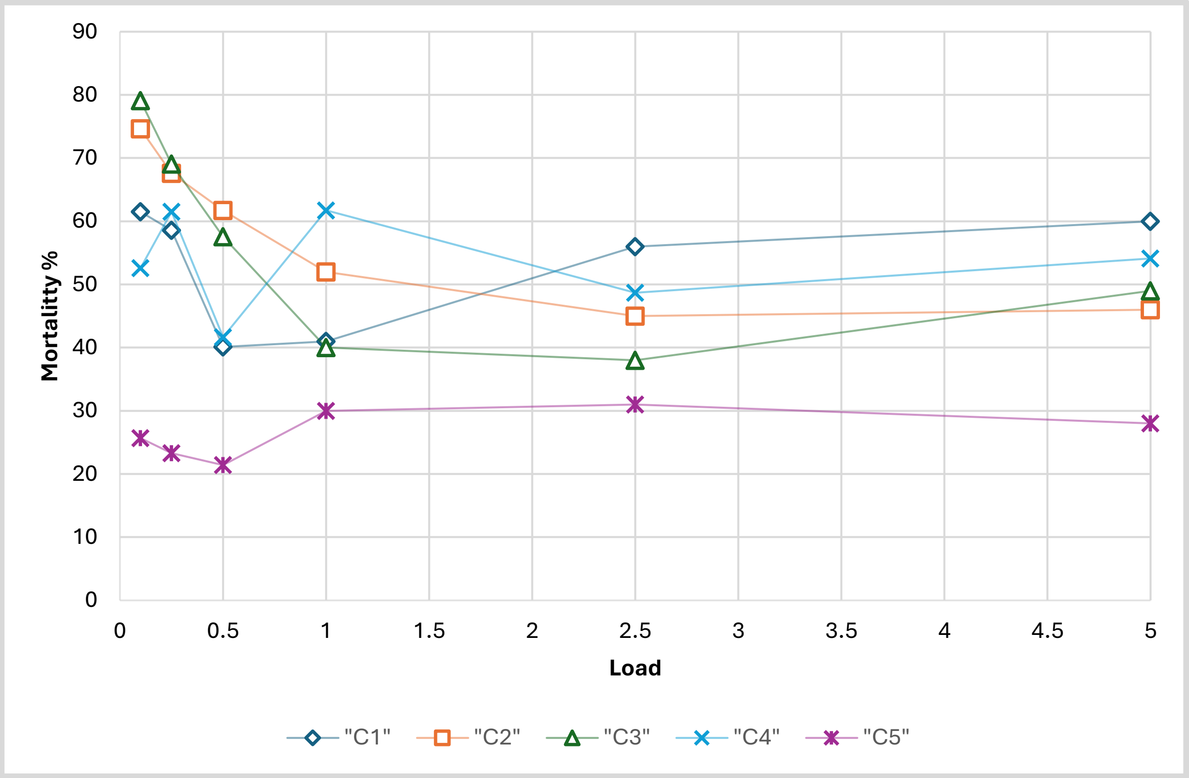

*Figure 10. Mortality for six different loads and five different concentrations*

For all concentrations other than C5, the larvae exhibited consistently high mortality rates, particularly in C1 to C4. As illustrated in Figure 10, C5 demonstrated superior performance across all organic load levels, both in terms of larval survival and weight gain. At lower organic loads (0.1, 0.25, and 0.5), the mortality rate for C5 remained relatively low, ranging between 20% and 25%. Even as the organic load increased to 1, 2.5, and 5, the mortality rate remained stable at approximately 30%, indicating that C5 provided a stable and supportive environment for larval development.

In contrast, the lower concentrations (C1 to C4) exhibited significantly higher mortality rates, with the most severe cases observed in C2 and C3, where mortality approached 80%. This trend suggests that these concentrations failed to provide the necessary conditions to sustain larval survival. Notably, C4 proved to be particularly inadequate, as mortality rates exceeded 40%, reinforcing the conclusion that it does not offer a viable environment for larval development. This indicates that the threshold concentration for larval survival lies between C4 and C5, with C4 being clearly insufficient.

The results suggest that a higher organic concentration correlates with improved survival rates, as observed in the consistent reduction in mortality at C5. This trend highlights the importance of maintaining a sufficiently high concentration to ensure optimal larval growth and survival. The findings further emphasize that while organic load variations have some impact on mortality, the concentration of the substrate plays a more critical role. Thus, C5 emerges as the most favorable condition for larval survival across different organic loads, whereas concentrations below C5 lead to progressively higher mortality rates.

### 3.3. Yield

A negative yield indicates that the larvae not only failed to grow but also lost weight, suggesting they were unable to sustain themselves and began starving, the yield values can be seen in Figure 11 . This was observed in all concentrations except C5, where the yield remained above zero. Even at C4, the process proved ineffective, making it unsuitable for wastewater treatment applications. While biomass recovery is a beneficial side effect, the primary goal remains wastewater treatment, and concentrations below C5 fail to support larval growth adequately. The highest concentration tested (C5) demonstrated the best performance, reinforcing the importance of maintaining sufficiently high substrate concentrations.

Regarding organic loads, yield tends to decrease at higher loads. At elevated loads, larvae have access to excess food, allowing them to allocate nutrients both for growth and for their metabolic activities. In contrast, at lower food availability, nutrients are prioritized for growth rather than for sustaining additional metabolic functions. When food is severely limited, larvae consume all available nutrients solely for survival rather than for growth. This explains why, in the first three organic load levels, growth is observed, whereas at higher loads, larvae must allocate resources between movement, maintenance, and growth, leading to a decline in yield.

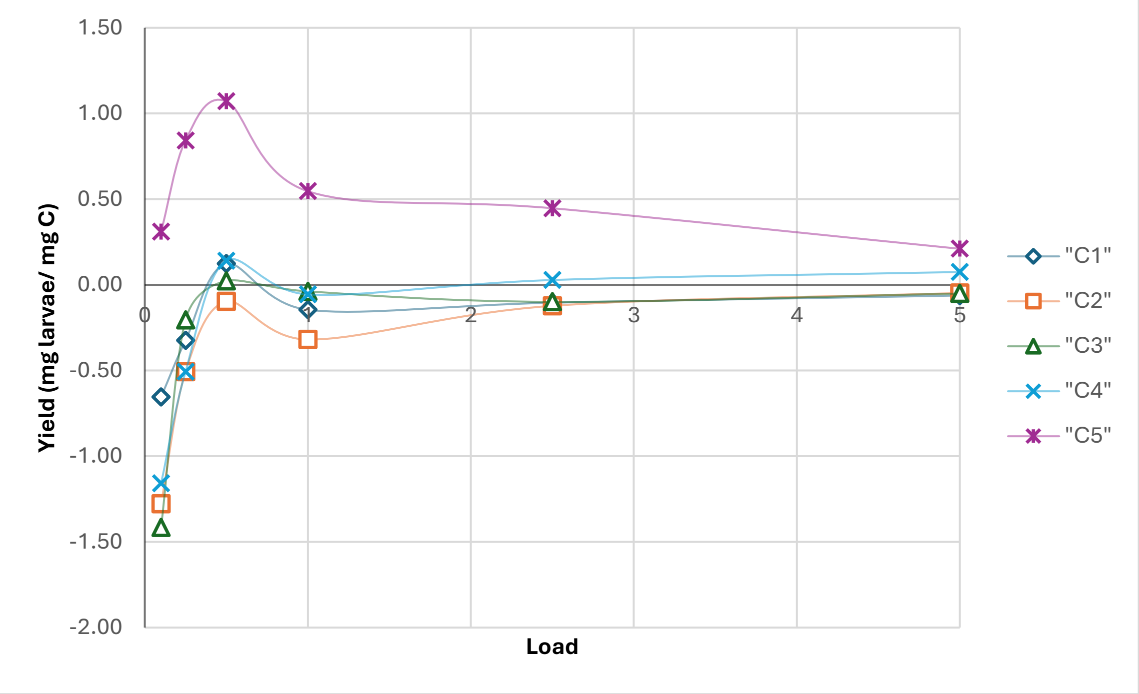

*Figure 11. Yield (mg larvae/ mg C) for five different concentrations and six different loads*

### 3.4. Specific Substrate Consumption Rate

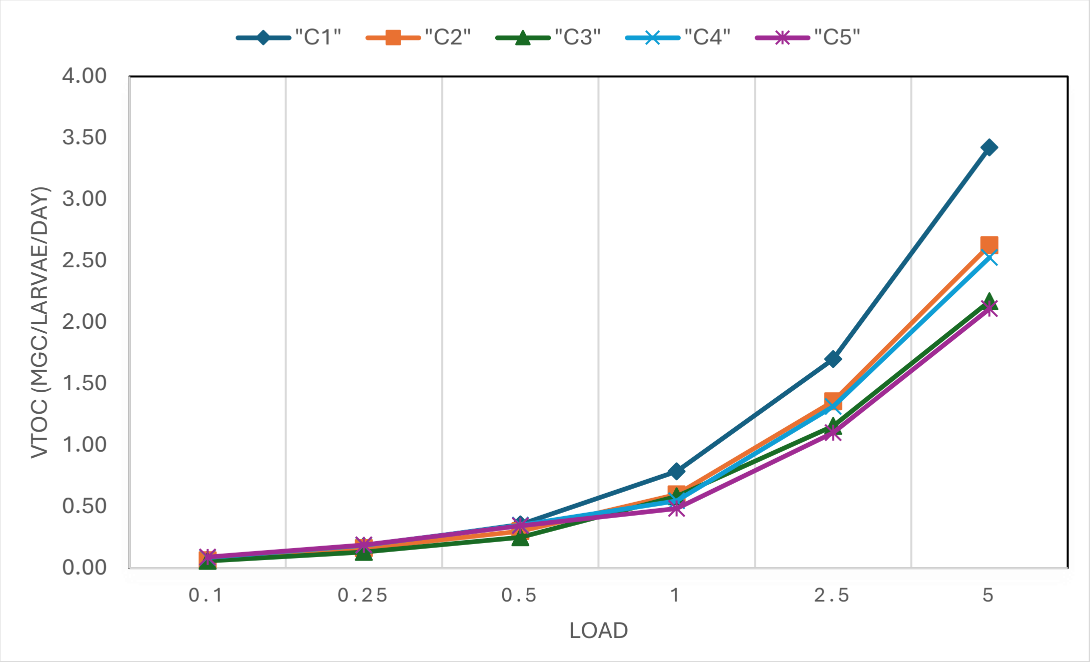

*Figure 12. Specific substrate consumption rate for six different loads and five different concentrations*

The results indicate that substrate consumption is primarily influenced by the organic load rather than the concentration. Regardless of the concentration, the same load follows a consistent trend, suggesting that the determining factor for substrate consumption is the load itself. Since all larvae receive the same type of substrate, variations in concentration do not appear to impact the specific substrate consumption rate (Vs).

As expected, all data points follow a similar pattern across different concentrations, reinforcing that concentration does not play a significant role in influencing substrate consumption. However, an exception is observed at C1, where a deviation occurs. This anomaly may be due to the presence of microorganisms contributing to aerobic digestion, though the low concentration makes it difficult to determine a clear justification for this behavior.

The primary objective remains to identify the threshold concentration at which the process becomes effective, ensuring optimal conditions for larval performance and substrate consumption.

## 3.5. Efficiency

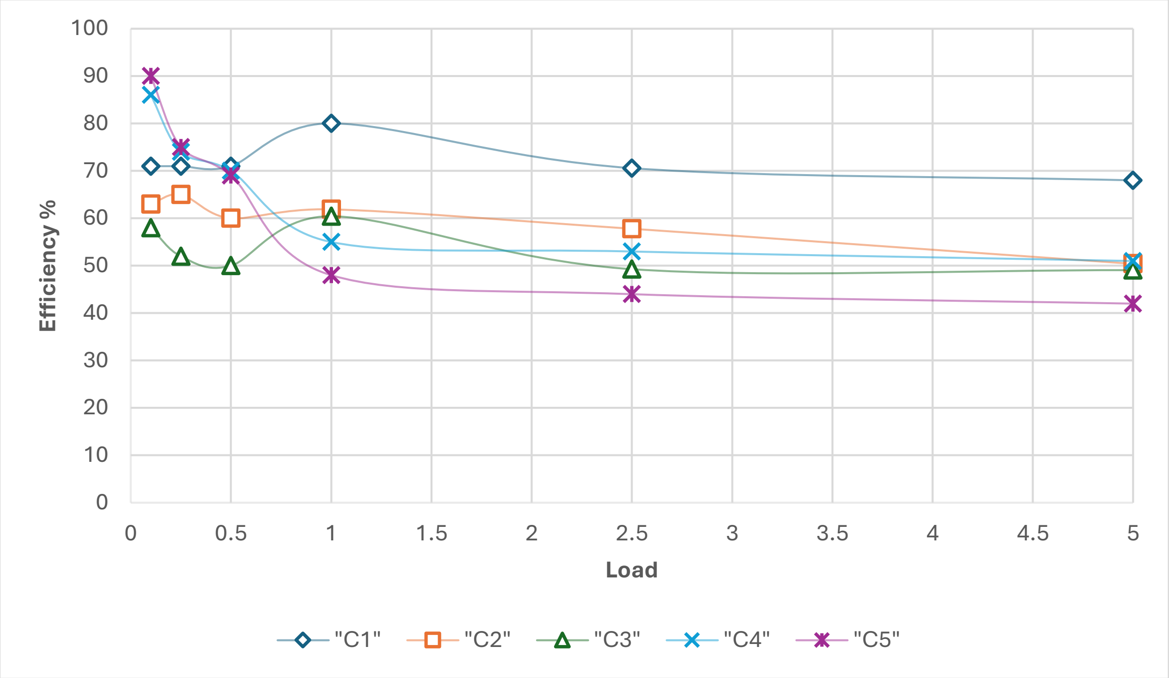

*Figure 13. Substrate conversion efficiency for six different loads and five different concentrations*

The removal efficiency data indicate that for C4 and C5, that can be seen in Figure 13, which represent more concentrated diets, the efficiency remains relatively stable. Both concentrations follow a similar trend, suggesting that a higher substrate concentration supports consistent larval performance. In contrast, C1, C2, and C3, which are lower concentrations, exhibit significantly lower removal efficiencies. These concentrations fail to support the process effectively, as the larvae are unable to perform optimally under such conditions. The system does not behave as expected at these lower concentrations, further reinforcing that only at C4 and C5 do we observe a stable and predictable removal efficiency.

Notably, removal efficiency reaches a steady state beyond an organic load of 1, remaining relatively constant across higher loads. This consistency is particularly valuable for the design and scaling of a pilot plant. Since efficiency does not decline with increasing load, it is possible to increase the organic load while reducing the reactor volume without compromising performance. In practical terms, this means that whether the plant is loaded with an organic load of 1 or 5, the removal efficiency remains the same. Operating at higher loads, such as 5, offers a significant advantage, as it allows for a more compact reactor design while maintaining the desired treatment efficiency. This finding is crucial for optimizing system design, ensuring both operational efficiency and economic feasibility in larger-scale applications.

## 4. Conclusions

This study highlights the significant impact of both substrate concentration and organic load on the performance of Black Soldier Fly (BSF) larvae in wastewater treatment. The results show that concentration is the primary driving factor for process efficiency, with C5, which is the highest concentration, consistently outperforming lower concentrations in terms of substrate consumption, growth, and removal efficiency. In contrast, concentrations below C4 led to high mortality rates, negative yield, and inefficient organic matter removal, demonstrating that a threshold concentration above C4 is essential for successful treatment.

Substrate consumption trends revealed that organic load, rather than concentration, is the key determinant of larval metabolic activity. Regardless of concentration, larvae followed a similar substrate consumption pattern at the same load levels, indicating that total organic availability plays a crucial role in the process. However, an exception was observed at C1, where an unusual trend appeared, the extremely low concentration made it difficult to draw definitive conclusions.

The yield results showed a clear pattern in terms of, at higher loads, larvae having sufficient food not only for growth but also for metabolic maintenance, leading to a decline in yield after the first three loads. When food availability was low, larvae prioritized growth over metabolic activity, but when the organic load increased beyond a certain point, they allocated nutrients to both growth and metabolic activity, causing a reduction in biomass yield. This suggests that the optimal load selection is necessary to balance biomass production with overall treatment efficiency.

Removal efficiency data shows the advantage of higher concentrations, particularly C4 and C5, where efficiency values remained stable across increasing organic loads. In contrast, lower concentrations (C1–C3) exhibited poor process performance. Importantly, removal efficiency remained relatively constant at higher loads, which presents a significant advantage for scaling up the process to the pilot plant. This stability allows for an increase in organic load while reducing reactor volume without compromising treatment performance, making the system more efficient for large-scale applications.

A key trade-off emerged between treatment efficiency and biomass recovery. At low loads (below 1), removal efficiency was high, reaching up to 90%, but biomass generation was minimal, limiting the potential for resource recovery. Conversely, higher loads resulted in increased biomass production, but with lower removal efficiencies (around 50%). This suggests that the process can be chosen based on specific objectives, prioritizing wastewater treatment for high-efficiency organic removal or balancing treatment with larval biomass recovery for resource utilization.

From an application perspective, this technology is best suited for treating high-strength wastewater, where larvae exhibit enhanced substrate consumption and growth. The process could serve as an effective pre-treatment step. Additionally, considering that BSF larvae naturally prefer solid feed, higher concentration waste streams would likely yield even better performance, bringing the system closer to the larvae's optimal feeding conditions, which is a solid environment. Eventually, this technology could be further optimized for super-concentrated wastewater applications, where both efficiency and biomass generation are maximized.

To conclude, the use of BSF larvae for wastewater treatment suggests a promising, sustainable solution, particularly with high organic concentrations. The ability to maintain stable removal efficiency at high loads provides an opportunity for optimizing reactor design, reducing system footprint, and improving cost-effectiveness.

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