UNIVERSITÀ DEGLI STUDI DI PADOVA
DIPARTIMENTO DI INGEGNERIA CIVILE, EDILE E AMBIENTALE
Department of Civil, Environmental, and Architectural Engineering
Corso di Laurea Magistrale in Environmental Engineering

MASTER THESIS

WASTEWATER TREATMENT USING BSF LARVAE: INFLUENCE OF LARVAE DENSITY AND SOLID SUPPORT

Supervisor: ING. PROF.SSA VALENTINA GROSSULE

Student: RACHELE POSSANZINI 2024144

ACADEMIC YEAR 2021-2022

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I would like to thank Prof.ssa Valentina Grossule for giving me the opportunity to work on this fascinating project, for her guidance, support and help during the work. I would like to thank also Doct.ssa Annalisa Saldon for her technical support during the samples analysis, but also for her psychological support. Finally, I would like to thank all the family of Voltabarozzo Lab which has welcomed, entertained, and supported me during these months.

I dedicate this thesis to my family and friends who supported me during all my studies.

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ABSTRACT

Recently the biological treatment of wastewater with the use of Black Soldier Fly (BSF) larvae has been studied with promising results, particularly in the case of high organic content (HOC) wastewater, such as sewage from food industries, leachate from municipal solid waste (MSW) landfill, etc. The use of a physical support for the larvae mobility has been suggested and patented to overcome the issue of high larvae mortality under pure liquid conditions. Moreover, the process influencing variables and removal kinetics have been studied under different HOC wastewater, providing design parameters for real scale treatment plants. For the scaling up of the process, the definition of type of physical support and larvae density are still to be investigated. The aim of this study was to achieve 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. Three distinct supporting materials (Valox® plastic granules, Kaldnes® and geomat) were tested at four different larvae densities (4, 8, 16 and 32 larvae/cm2). The larvae were fed with the same artificial wastewater (low-fat milk diluted with water, 1:10) under same organic load. The biological treatment performance was assessed by monitoring both the larvae growth (in terms of weight variation and prepupation) and Organic Carbon removal performance.

1 INTRODUCTION

The treatment of high organic content wastewater, aimed at meeting the increasing quality standards set by the regulators, typically requires great technological and economic investments. Consequently, there is growing interest internationally in developing, within the concept of the Circular Economy, simple cost-effective technologies inspired by nature, aimed at the recovery of viable energy and material resources. In the context of the Circular Economy, an interesting alternative is represented by the Black Soldiers Fly (BSF) larvae, which are capable of metabolising and stabilising huge amounts of organics, transforming it into valuable biomass rich of proteins and fats, suitable to be used respectively for animal feeding and biofuel production.

BSF larvae have been wildly used for solid biowaste treatment, offering a proper environment for larvae, while the research on the use for liquid substrates treatment is still at the very beginning. This process has recently been tested on MSW landfill leachate with encouraging results. Grossule et al. (2020) and Grossule and Lavagnolo (2020) demonstrated the capability of larvae to metabolise liquid substrates producing a good quality prepupae biomass, without significant inhibitory effects caused by toxic compounds in leachate. The main critical issue resulting from these studies was the relatively high mortality occurring under pure liquid conditions due to larvae drowning. The issue was, at first, tried to be solved by adding solid organic substrate, but it failed due to the preference of larvae to feed on solid substrate rather than on liquid substrate with the consequence of not treating the interested liquid substrate. Considering this, it was chosen an inert material, plastic granules (Valox®), which was able to provide the requested physical support by larvae without compromising the targeted treatment of the liquid substrate (Grossule and Cossu, 2021).
The encouraging published results demonstrate the capability of larvae to growth on liquid substrates, but the research is still facing critical aspects:

a) Substrate quality
b) Removal efficiency

c) Organic load vs. concentration
d) Feeding approach
e) Larvae density.

The a) issue was solved by Grossule, Fang, Yue, & Lavagnolo, (2022), obtaining that the quality of the organics in the substrate affects the treatment performance: the larval growth, survival rate and prepupation increase proportionally to BOD_5/TOC ratio; in that same study the point b) was investigated with the results that higher organic removal efficiency is reached, achieving up to 3-fold higher specific substrate consumption rate (mgCOD/mgVS/d) compared to values reported in literature for activated sludge.

The study of Grossule et al. (2022) was aimed at a better understanding of the c) aspect, gaining the following conclusions: below the substrate load of 10 mgC/larva, the substrate concentration in the feeding (mgC/L) was not influencing larval growth (in terms of maximum wet weight, prepupation and mortality), on the contrary, above 10 mgC/larva, the influence was quite significant, higher the load, higher the positive impact of the substrate concentration.

The d) and e) critical aspects are the goal of the present work: three different supporting materials (Valox®, Kaldnes® and geomat) are tested at four distinct larvae density (4, 8, 16 and 32 larvae/cm^2). The aim of this study is to achieve 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.

1.1 BSF larvae: role in Circular Economy

In the framework of the Circular Economy, in which the elimination of waste and pollution is one of the three principal pillars, the use of Black Soldiers Fly (BSF) may play an important role managing the waste and providing resources in term of materials and energy (Grossule & Lavagnolo, 2020). Hence, their application in solid waste management is making itself a name as a very efficient green technology. This species of fly has the capability to reduce consistently huge amounts of organic biomass and concurrently can offer valuable animal or human feed having nutrient composition. In fact, these larvae can metabolise and stabilise putrescible waste, transforming it into valuable biomass rich of proteins and fats, appropriate to be used for animal feeding and biofuel production. BSF has been already used in different kinds of biowaste, such as food waste, dairy manure, poultry waste, kitchen waste, agricultural residues and human faeces (Singh & Kumari, 2019). Therefore, BSF larvae contribute to the recycle of waste and to the reduction of its environmental footprint, embracing the principles of Circular Economy.

Black Soldier Fly (Hermetia illucens) is one of the most extensively distributed species among the sub family Hermetiinae. These flies are native of America, in particular of Hawaiian Islands; they are spread in tropical and warm temperate regions (between about 45°N and 40°S) (Singh & Kumari, 2019).

The life cycle of the BSF, depicted in Figure 1, consists of five stages: eggs, larvae, prepupae, pupae and adults. The maximal contribution of the entire life cycle is given by the larval and prepupal stage whereas the other stages are relatively short. In fact, only during the larval stage, these flies feed as much as they can, and once they pupate and transform into adults, they stop feeding and rely only on their fat reserve of the body. This feature of feeding habits limits the risk of disease transmission (Diener et al., 2009)(Sheppard et al., 2002) (Banks et al., 2014). The life span of the larval stage is the longest, which can take about 2-4 weeks, but can be prolonged up to several months if conditions are unfavourable. The prepupal and pupal stage last respectively 7 days and 10 days to months, while the one of the adult flies is just between 5 to 8 days. The end of the larval stage and thus the start of the prepupal stage is characterized by a change in colour of the larvae; in fact the white colour becomes darker and they self-harvest by leaving the wet feeding for a drier site (Banks et al., 2014) (Tomberlin et al., 2009).

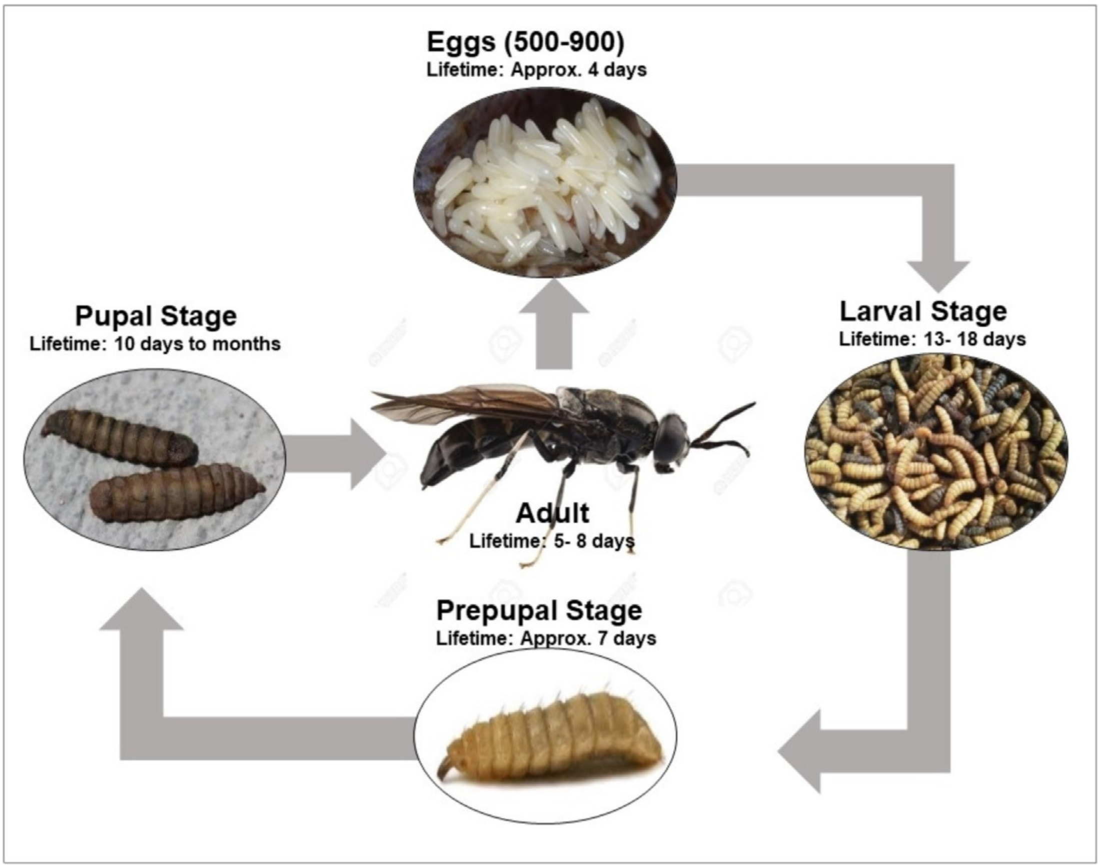

Figure 1: Typical lifecycle stages of Black Soldier Fly (Singh & Kumari, 2019)

Despite the fact that BSF can adapt themselves in many different environmental conditions, food shortages or oxygen deficiencies, presence of insecticides and pesticides, and that can compete with other flies development (Makkar et al., 2014) (Turchetto & Vanin, 2004), the environmental conditions and food quality/quantity heavily influence their success and time of development (Grossule & Lavagnolo, 2020). In particular the optimal conditions are reported to be: moisture between 30-80%, temperature ranging from 25 to 27 °C, photoperiod 18 light / 6 dark (hours), pH>6 and food quantity and quality 125 mg/larva/ d of biowaste (Tomberlin et al., 2009) (Diener et al., 2009) (Zurbrügg et al., 2018) (Sheppard et al., 2002) (Popa & Green, 2012).

Even if the BSF larvae develop the most in the previous reported environmental conditions, these larvae are robust, tolerant, and flexible to adapt themselves making this treatment technology unsophisticated and of easy operation. It is the one such technology that integrates three major terms waste composting, nutrient recovery, and income generation in its application (Sheppard et al., 1994). The BSF larvae treatment shows several of social, economic and environmental benefits, among which massive organic waste reduction is the principal and sustainable means of solid waste management. Diener et al., (2011) report that 65-78% of total waste reduction upon the amount of waste added daily, while Pathak et al. (2015) in Indian climatic conditions, feeding on kitchen waste, assess a waste reduction of 50% of original volume. In addition, the larvae increase their biomass up to 20% dry matter, and the prepupae (44% dry matter) contain 36-48% protein and 31-35% fat (Banks et al., 2014; Diener et al., 2009; Sheppard et al., 2002). These features make the BSF larvae a rich source of proteins and fats which can be used as animal feeding food, and the high lipid content present can be exploited by the biofuel industry for its production. In fact, the transesterification of the fat extracted from the BSF pupae led to the production of biodiesel with good fuel properties (e.g., Viscosity, density, flash point, cetane index) meeting the European biodiesel standards (EN14214) (Li et al., 2011). A farther benefit is the nutrient residues generated after the decomposition of the organic waste which can be used as fertilizers (Singh & Kumari, 2019). An additional discovery made by different authors is the ability of the BSF larvae to modify the microflora present in the waste while composting and to reduce bacteria such as F. coli and Salmonella enterica by secreting harmful bactericidal compounds (Erickson et al., 2004; Gabler, 2014; Lalander et al., 2015). All these BSF application, organic waste composting, faecal bacteria reduction, biofuel production, organic manure, animal feed and feed for aquaculture, make the species to be called "the dynamic creature" (Singh & Kumari, 2019).

Insect biotechnology is obtaining big attention nowadays and the Black Soldier fly, H. illucens, is one of such successful insect biotechnology. The species can grow and harvested without any dedicated facilities or high operational skills; their ability to convert waste into food, generating value and closing nutrient loops as they reduce pollution and cost (Wang & Shelomi, 2017), make them environmental sustainable obtaining a role in the context of Circular Economy.

1.2 The use of BSF larvae in wastewater treatment: from first studies to the patent

As previously said, the BSF has been successfully applied to different kinds of waste, a wide range of decomposing organic matter such as spoiled feed, kitchen waste, animal manure, etc. Although their evident benefits in treating organic waste, the species was never applied to wastewater or leachate, characterized by a high organic content. The very first study, currently known, using BSF larvae on wastewater treatment is the one of Popa and Green 2012; they have been used for the first time BSF larvae for the treatment of leachate. The results of their study were very promising, pointing out that those larvae were able to reduce COD and volatile fatty acids (VFAs) while growing within the leachate.

After this first study, Grossule & Lavagnolo (2020) had investigated the adaptability of BSF larvae to leachate treatment, testing at lab-scale different percentages of leachate (from 25 to 100%) to the feeding liquid, two different substrate conditions (liquid media and semisolid media mixing liquid with wheat bran bedding). Many parameters were investigated: wet weight of larvae variation over time, larvae mortality, time required to achieve the prepupal stage, percentage of prepupation, and protein and lipid contents, including profiling of fatty acids. In each test 200 mL of liquid was placed, and just in the semi-solid tests 50 grams of wheat bran were added; the feed was replaced twice every week. The results obtained were very promising; they showed that the BSF larvae grew under all different feeding configurations but with different patterns. The larvae supported on wheat bran exhibited a faster and higher development compared to the ones growing just on liquid due to the additional food provided by the bran. For this last reason no significant differences were reported in the various leachate concentration. The maximum larvae weight ranged between 205-253,7 mg w/w and after 24 days the tests ended due to the reaching of the prepupal stage. Instead, in the liquid configuration the leachate concentrations (25, 50, 75 and 100%) strongly influenced larvae development, in fact just in the leachate concentration of 100 % the prepupation was achieved after 35 days within the test period. In the 25, 50 and 75% the larvae gained a wet weight in the range of 55.5-66.6 mg/larva, while in the 100 concentration the maximum larvae weight was 119.7 mg w/w. Apart from weight analysis, the mortality was studied: in bran-supported boxes it varied between 10 to 14 %, while the highest mortality (40%)

was observed in the most diluted feeding under liquid substate condition (25%). The Figure 2 summarizes the results regarding the larvae development and the mortality. From the prepupal development, it was attested that the positive effect produced by the leachate with no signs of inhibition effects.

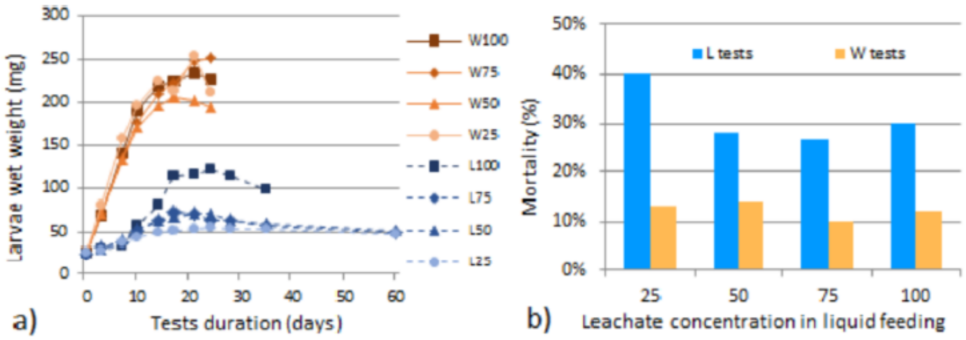

Figure 2: a) Variation of average larval wet weight under the different feeding conditions adopted (W=wheat bran substrate, L=liquid substrate, 25-100=percentage of leachate in the feeding liquid). b) Larvae mortality (% of dead larvae over the total number of larvae at the start) under the different feeding configurations. (Grossule & Lavagnolo, 2020)

The results relating to the characterisation of the dry mass accumulated by the prepupae during their development show similarities with data from other literature studies in which BSF larvae were fed on dairy manure (Li et al., 2011) and food waste (Surendra et al., 2016). The lipid content in prepupae grown up on wheat bran decreased as leachate concentration increased with ranges between 28.3% and 21.7%, while in the liquid configuration with 100% leachate it was 21.2%. Lipids were classified according to their saturation to predict the biofuel potentially produced. It can be state that the predicted quality of biodiesel from the prepupae grown on liquid substrate (100% leachate) is expected better than the one produced from wheat bran supported prepupae. Also, the protein content in prepupae coming from wheat bran tests decreases as the leachate concentration increases, with values between 50% to 46.4 %, instead in the case of liquid tests with 100 % leachate it reaches a value of 38.1 %. These values obtained differs from those reported in the literature (31.9-46.3%) (Barroso et al., 2014; Diener et al., 2009; Surendra et al., 2016). From these results regarding lipids and proteins, it is demonstrated that both are influenced by the feeding substrate and might be controlled by mixing leachate with different solid substrates. In conclusion Grossule & Lavagnolo (2020) evinced that: leachate has no significant inhibitory effect, higher larval development under liquid feeding conditions occurs in higher concentrated leachate, and among the two substrate conditions larvae prefer the semi-solid one.

After the assessment of the adaptability of BSF larvae to landfill leachate, it was necessary to investigate the treatment capacity of BSF larvae under various operative conditions. This step was performed in the study of Grossule et al. (2020) by mixing leachate with different kinds of solid supports including residues (wheat bran, brewers' spent grain and sawdust). During the test it was monitored: the larval growth (in terms of wet weight variation, mortality and prepupation percentage), the prepupal biomass composition (in terms of crude protein, lipids and fatty acid profile), the treatment performance (measuring variation of total solids, total organic carbon and nitrogen compounds) and the stabilisation (measuring the 7-day Respirometric Index) in each substrate. To evaluate the individual contribution of larvae in the leachate treatment, control tests without larvae were performed in parallel with the ones with larvae. The feeding was 100 mg substate/larvae/day as proposed by Diener et al.(2009), in practice the total amount of semisolid substrate added to each box was 600 g (120 g solid support + 480 g leachate). After the test period of 63 days, the results regarding the larvae development were obtained, demonstrating that the wheat bran and brewers' spent grain are the best solid supports. In fact, after only 21 days it was recorded an average larvae weight for wheat bran of 226.1 ± 5 mg (w/w) with a prepupation of 94%, while the values for the spent grain were a bit lower ranging between 148,3 and 161,9 (w/w) and 82%. For sawdust, prepupation was not achieved after 63 days of test duration, the average larvae weight was the lowest of 46.0 ± 2.9 mg (w/w). The results obtained are consistent with the different availability of degradable carbon and nutrients in the tested feeding substrates, wheat bran and spent grain are characterised by a high content of short-chain carbohydrates and proteins, providing carbon and nutrients further to leachate. 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 1, the removal efficiencies in each reactor containing larvae are higher compared to the one in the respective control reactor.

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
	RI7	-	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
	RI7	-	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
	RI7	-	36.5	13.5

Table 1: Wheat bran (W), Spent grain (B), Sawdust (S), Total solids (TS), total organic carbon (TOC), total nitrogen (TN), organic nitrogen (Norg), and 7-day Respirometric Index (RI 7) reduction efficiency in larvae box and in control box (Grossule et al., 2020).

Values around 60% were observed for TOC removal efficiencies for wheat bran and spent grain tests, while for sawdust test the value remained below 25% due to the low biodegradability of the support material. On the contrary, for TKN removal efficiencies sawdust tests showed higher % in respect to wheat bran and spent grain; these results are explained by the lower load of TKN in sawdust tests. However, the removal efficiencies of the larvae in the different substrates were related to the form of nitrogen in the TKN load. In sawdust tests the prevailing form of nitrogen was ammonia, whose removal by larvae was negligible ($\Delta\eta_{larvae}$= 2,6 %), while when Norg was the prevailing form, in the case of wheat bran and spent grain tests, the larval metabolism could remove it in a very efficient way. In fact Popa & Green (2012) obtained the same results, observing that BSF larvae were not able to metabolise nitrogen in the ammonia form. Anyhow ammonia nitrogen didn't appear to have any toxic effects for larval development. In all the tests larval activity contributed significantly to the stabilisation of the substrates, in particular the wheat bran tests showed a stability reached 2.6-fold higher than in the control box. Lipid and protein contents were evaluated in the prepupal biomass, obtained from wheat bran, and spent grain tests in which prepupation was reached; lipid and protein concentrations ranged between 27.9 and 29.8% and between 43.5 and 45.3% respectively. Lipids were classified also according to saturation classes, to predict the quality of the potential biofuel produced from prepupae. The results obtained indicate 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). The research concluded that the development and performance of larvae in removing contaminants is related to the test substrate, in particular wheat bran and spent grain supports provided nutrients other than leachate displaying the best performance.

The Grossule et al. (2020) study concluded that further studies should be performed to assert the sensitivity of the system to different quality of leachate and its fluctuations; further solid supports should be investigated, considering inert materials such as sand, plastic or sawdust. These two issues were analysed and solved in the study performed by Grossule et al. (2022), by using first an artificial leachate, purpose-designed to simulate real leachate and guarantee reliable, constant, repeatable and representative concentrations (Grossule, Fang, Yue, Lavagnolo, et al., 2022), and by adopting an appropriate set up of the reactor system, providing efficient physical support for larvae mobility (Grossule and Cossu, 2021). This study aimed to achieve a better understanding of the influence of the biodegradability and degree of oxidation of organic content on leachate treatment performance, defining organic removal kinetics needed to design a BSF larvae reactor for use in HOC wastewater treatment. Six different leachates, characterised as in **Errore. L'origine riferimento non è stata trovata.**, both real and artificial were used in the study; they have been selected and prepared ensuring similarity of COD concentrations and varying the degree of biodegradability and oxidation, represented respectively by BOD5/COD and TOC/COD ratios.

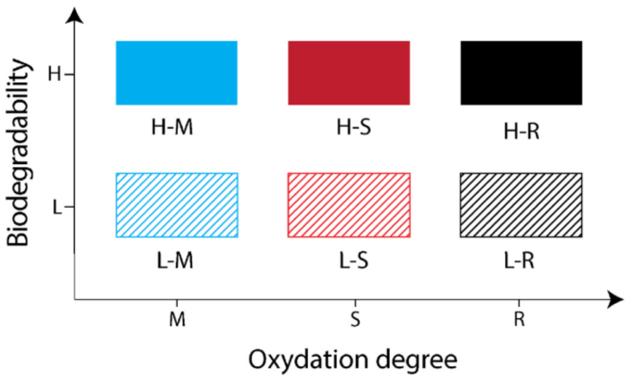

Figure 3: Graphical description in terms of biodegradability and oxidation degrees of the six leachates tested in the research programme. (R= real leachate; S= Synthetic artificial leachate; M=mixed artificial leachate; H= high biodegradability; L= low biodegradability). (Grossule, Fang, Yue, & Lavagnolo, 2022)

Variation of larval weight over time, larval mortality, and percentage of prepupation, growth and substrate removal kinetics, average daily TOC and VFA consumption, larval protein and lipid contents with the profile of fatty acids were analysed to evaluate the BSF larvae process performance. Young BSF larvae were placed in batch reactors with patented plastic granular bed, which was fully saturated by leachate. The analytical composition of the tested leachates, graphically showed in

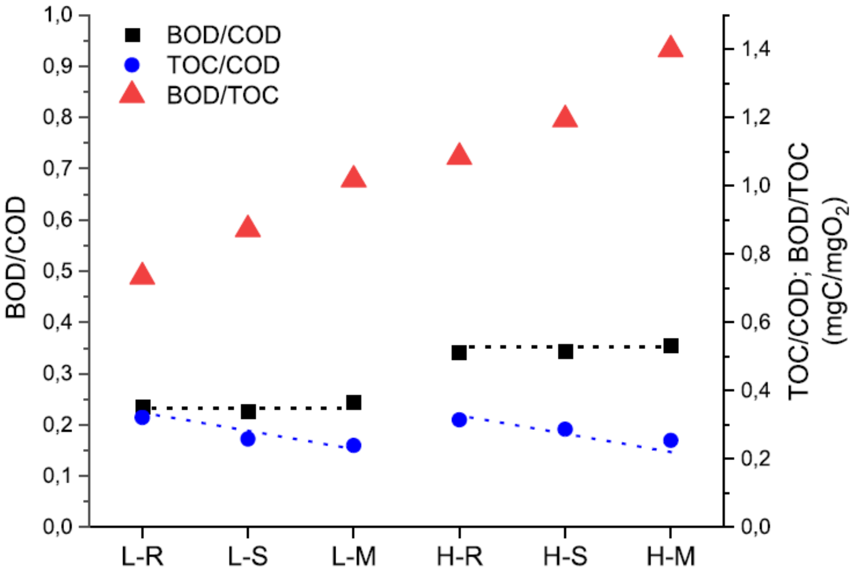

Figure 4, presented: similar COD concentrations of 12,000-13,000 mg/L, BOD_5 between 2900 mg/l and 4500 mg/L for low and high biodegradability leachates respectively, an average BOD/COD ratio of 0,24 (low biodegradability leachate, L) and 0,35 (high biodegradability leachate, H), different TOC values according to real and artificial leachates, decreasing from real (R) to artificial synthetic (S) and artificial mixed (M) leachate, TOC/COD ratio significantly divergent between real and artificial leachates independently from the degree of biodegradability, organic nitrogen (N_{org}) lower in mixed leachates and higher in synthetic leachates, and a Biotreatment Index represented by the ratio BOD/TOC varying significantly in a linear manner, representing the combination of biodegradability and oxidation variations. As in the previous studies the tests lasted till 60 days, with constant feeding conditions.

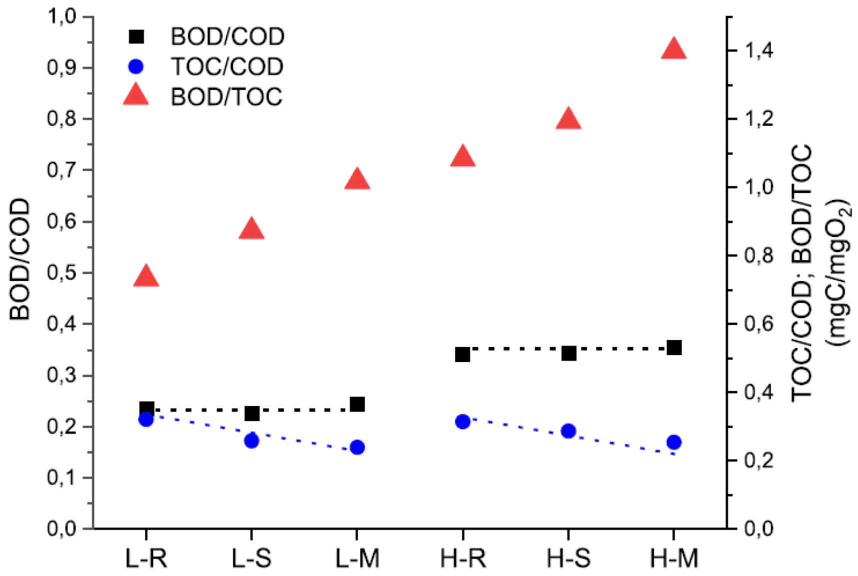

Figure 4: Graphical representation of TOC/COD, BOD_5/COD and BOD_5/TOC values of all tested leachates (R= real leachate; S= synthetic artificial leachate; M= mixed artificial leachate; H= high biodegradability; L= low biodegradability). (Grossule, Fang, Yue, Lavagnolo, et al., 2022)

Regarding the analysis of the variation of average larval wet weight (X, mg/larva) over time (t,days) the model adopted was the Verhulst logistic growth model, which appeared to be the best fit. The maximum biomass wet weight reached (X_{max}) in the growing phase, and the maximum specific growth rate (the μ_{max} day^{-1}) were proportional to the BOD_5/TOC ratio in all tested leachates. In fact the highest X_{max} and μ_{max} corresponding to 85 mg/larva and 0.24 d^{-1} respectively, were achieved at the highest BOD_5/TOC (1.40 mgC/mgO_2), while the lowest μ_{max} (around 0.05 d^{-1}) was observed at the lowest BOD_5/TOC (0.73 mgC/mgO_2). Also, mortality and prepupation confirmed the relationship between growth performance and variation of BOD_5/TOC. Mortality values ranging between 32 to 5% obtained under BOD_5/TOC values of 0.73 and 1.40 mgC/mgO respectively, showed an inversely proportional manner to the Biotreatment Index, while prepupation showed a proportional manner, remaining below 4% in low biodegradable leachates and varying from 10 to 21% in high biodegradable ones. Besides, for low biodegradable leachates, the growth rate r_x (mg larva/d) showed a lower and less rapid development compared to high biodegradable ones, and between these latter (with same level of biodegradability) better growth performance was observed for leachates characterised by a lower degree of oxidation, i.e., higher for artificial mixed leachate, lower for real leachate. Also Laganaro et al. (2021) obtained that the specific growth rate and the growth rate were negatively affected by the substrate quality. The yield (Y, mglarva/mg TOC), calculated at the 6th day when the max growth rate occurred, varied significantly between the different leachates, attesting the occurrence of a higher conversion of substrate into new biomass proportional to higher BOD_5/TOC. After the 6th day, yield decreased in all tests, reaching the same final values at the end of the growth phase. These results attested that, despite the same COD concentration, larvae metabolism appeared to be clearly influenced by the degree of biodegradability and oxidation, in practice by the quality of organic content of the treated wastewater. The calculated values of the maximum specific substrate consumption rates (k_{TOC} , $k_{COD, VS}$), reported in Table 2 , showed that mgTOC/mglarva/d (k_{TOC}) was almost independent of BOD_5/TOC unlike the efficiency of TOC conversion into new biomass (measured as Y) which changes in line with BOD_5/TOC. The substrate consumption rates values ranged between 0.49 and 1.37 mgCOD/mgVSlarva/d ($k_{COD, VS}$); if compared to data from conventional activated sludge treatment, the lavae metabolism proved to be more efficient in metabolizing biodegradable organics in respect to conventional activated sludge. In fact in the study of Tamrat et al. (2012) on MSW leachate treatment by conventional activated sludge the $k_{COD,VS}$ reported was of 0.35 mgCOD/mgVSlarva/d using a real leachate characterised by COD of 42,310 mg/L and a TOC/COD ratio of 29.

Test code	BOD_5/ TOC	TOC/ COD	K_{TOC}	$K_{COD,VS}$
			mgTOC/mglarva/d	mgCOD/mgVSlarva/d
L-R	0.73	0.32	0.051	0.49-0.99
L-S	0.87	0.26	0.062	0.75-1.51
L-M	1.01	0.24	0.060	0.78-1.56
H-R	1.08	0.31	0.050	0.49-0.98
H-S	1.19	0.29	0.063	0.68-1.37
H-M	1.40	0.25	0.056	0.68-1.37

Table 2: Maximum specific substrate consumption rate observed for the different leachates. (Grossule, Fang, Yue, Lavagnolo, et al., 2022)

As μ, Y and k, commonly used for designing leachate treatments, are strongly influenced by larval ageing, a new parameter was introduced, the specific daily substrate consumption, v_s, based on initial larval biomass (X_0) for designing BSF larvae reactors. This parameter, calculated both in terms of TOC (v_{TOC}) and VFA

(v_{VFA}), generally increased in line with growth of larvae, meaning that substrate consumption increased with aging of the larvae in the larval stage. Both average v_{TOC} and average v_{VFA} increased proportionally to BOD_5/TOC ratio, reaching a maximum value in high biodegradable mixed leachate test of 2.44 mg/TOC/larva/d. This further has proved that the best results are obtained at high degree of biodegradability (high BOD/COD) and low degree oxidation (low TOC/COD) of the liquid substrate. The last results were related to the characterization of the dry biomass of prepupae in high biodegradable tests, the only ones considered; no significant differences were detected in larvae protein and lipid contents, including the profiling of fatty acids. The research certified that the innovative feeding approach of mixing leachate with granular plastic material promoted an increase in the survival rate of larvae compared to data present in literature in all tests and that the higher the BOD_5/TOC ratio in the feeding substrate values, the greater the process performance.

Since in previous cited study, Grossule, Fang, Yue, & Lavagnolo (2022) investigated how biodegradability and degree of oxidation of organic content influenced treatment performance, the influence of organics load and concentration on the treatment process remained to be clarified. Therefore in the study of Grossule et al. (2022) it was focused on the better understanding of how the organic content load and concentration might affect the treatment performance. Low (L), medium (M), and high (H) concentrations artificial leachates were prepared, in terms of TOC, COD, BOD and Organic Nitrogen, and used for feeding with the same volume 20,40,80 and 160 larvae, thus testing four different loads. Both larvae growth (in terms of weight variation, mortality and prepupation) and leachate quality and quantity variation (to determine the substrate consumption) were monitored to assess the treatment efficiency. As in the study of Grossule, Fang, Yue, & Lavagnolo (2022) the experiment was done by placing young larvae in batch reactor filled with a patented plastic granular bed fully saturated by leachate. The analytical composition of the three tested leachates was performed and in all organic parameters, concentrations increased from L to M and H with a ratio apprx. 1:2:4, except for ammonia (approx. 1:2:2). The ratios BOD/COD, TOC/COD and BOD/TOC were similar in all the tested leachates so that the same quality of organics was guaranteed and they were comparable to those of the tests performed in Grossule, Fang, Yue, & Lavagnolo (2022). As it can be seen in **Errore. L'origine**

riferimento non è stata trovata., generally larval growth and TOC variation was higher and occurred faster as the organic content concentrations increased (from L to M and H leachate).

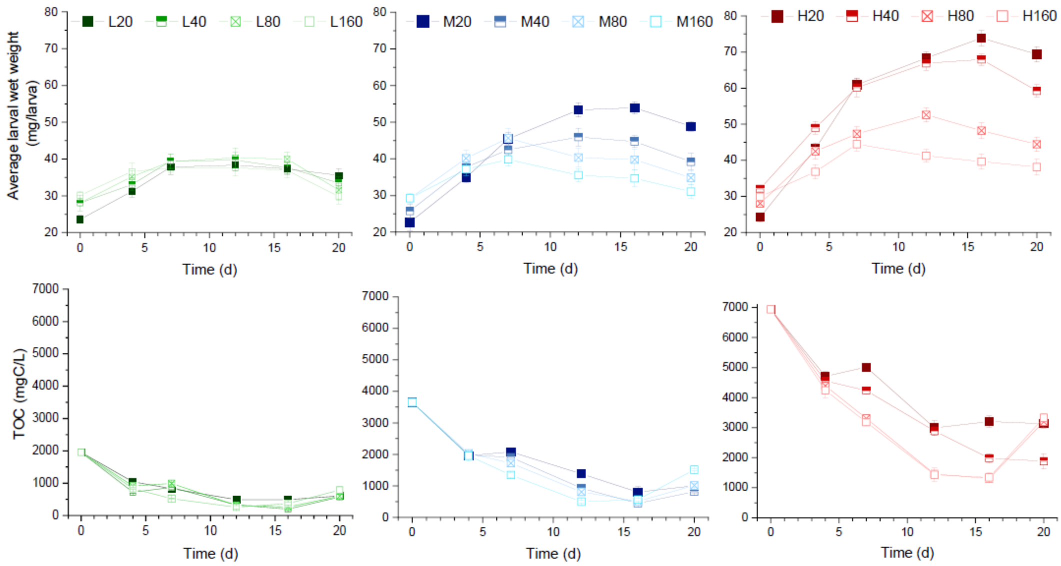

Figure 5: Variation of average larval wet weight (w/w) and substrate concentration (measured as TOC) under different leachate feeding typologies (L= low, M= medium, H= high-organic content concentration) and larval densities (20,40, 80, 160 larvae)(Grossule et al., 2022)

Among the L tests, larval growth and TOC variation showed similarities between the different larval densities (organic load): since the substrate concentration was very low, larvae starved under all feeding loads. On the 7th day the larvae reached the maximum wet weight of 39 ± 1.2 mg (w/w) and accordingly TOC concentration decreased significantly below 1000 mgC/L. While at the 17th day, when larvae started losing their weight and thus stopped their feeding, TOC concentration started to increase. On the contrary in M and H tests, the larval densities and therefore organic load significantly affected larval growth and substrate consumption. As expected, higher larval densities (lower organic loads) produced greater and faster TOC variation, but with a lower larval growth and an earlier achievement of max wet weight. The M80 and M160 tests achieved the max wet weight at the 7th day, while M20 and M40 at the 16th and 12th day respectively. Also, for H20 and H40 tests larvae growth stopped at the 16th day, rather than at days 12 and 7 respectively for the tests H80 and H160. At the end of the experiment, all tests showed a decrease of the larvae wet weight due to two possible phenomena: starvation, when TOC concentrations were below 1000 mgC/L, or the beginning of prepupation,in the case of H20 and H40 tests. Regarding the relationship between growth performance and substrate concentration and loads, in general it was deduced that organic content concentration had a greater effect on larval growth as the substrate load increased, influencing maximum wet weight, mortality and prepupation. TOC loads below 10 mgC/larva (L40, L160, L80, M80, M160, H160) produced lower max larval wet weight (between 35-45 mg/larva), lower prepupation percentages (between 0 and 2%), and higher mortality percentages (between 2,5-7%), regardless the substrate concentration. While in the case of higher TOC loads (>10 mgC/larva), significant differences were reported in max larval wet weight among the kinds of leachate as TOC loads increased, which can be visualized in

Figure 6: Variation of max larval wet weight with TOC loads, under different substrate concentrations. (Grossule et al., 2022)

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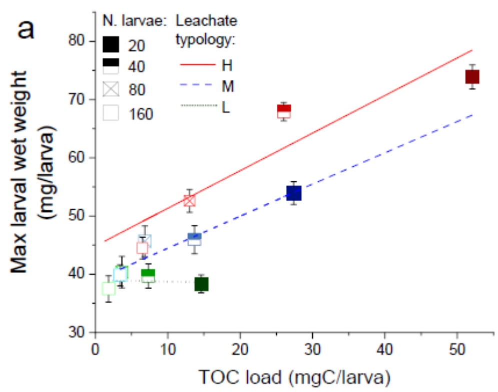

Figure 6: Variation of max larval wet weight with TOC loads, under different substrate concentrations. (Grossule et al., 2022)

Only in H tests prepupation increased as the substrate loads increased, achieving a value of approx. 17% in H20, consistently mortality decreased as the substrate loads increased but only in M and H tests. Regarding yield values, calculated for the growth phase, principally they decreased as the substrate loads increased, expressing that under food shortages, most of the available food is used for anabolic processes and converted into new biomass. The variation of yield values with the substrate loads was significantly influenced by the organic content concentration. Conversely to the larval growth rate, the organic substrate concentration didn't affect the substrate consumption. In fact, the specific daily substrate consumption values (v_s, mgC/larva/day) linearly increased as the organic substrate loads increased for each leachate typology and whatever the substrate concentration. In conclusion substrate concentration influenced the larvae growth and thus the potential amount of recoverable larvae biomass, but it didn't influence the specific daily substrate removal performance. Therefore, the v_s values could be used for treatment unit design, based on organic loads, regardless the organics concentration. From the research it was also evinced that TOC values approx. below 1000 mgC/L are limiting concentrations for BSF larvae process, making the use of larvae treatment appropriate for the pre-treatment of high organic concentrated wastewater.

From these reported studies, the patent regarding the use of Black Soldier Fly larvae in wastewater treatment has been filed.

1.3 The missing step from patent to real scale application

It has been proven 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, removing efficiently the organic substrates and converting them into a valuable protein and fat-rich biomass suitable for resource recovery. Moreover, the process influencing variables and removal kinetics have been established for different HOC wastewaters, providing design parameters for real scale application. But what are the limits of the former studies in the event of a real scale application of the BSF larvae treatment process? As demonstrated by Grossule & Lavagnolo (2020) the larvae fed just on liquid substrates achieved the highest % of mortality compared to those fed on semisolid substrate. Thus in Grossule, Fang, Yue, & Lavagnolo (2022) the tests were performed by adding solid inert support within the reactors. The BSF larvae were placed inside boxes filled by patented plastic granular bed (Valox) fully saturated by leachate, in this way the granular material allowed larvae to move freely to meet their needs (feeding and breathing). To saturate this supporting material 150 mL of leachate was used in a box of a volume of 600mL, and the remaining volume was occupied by the patented plastic granular bed. Even if this material showed great support to the larvae development and therefore to the success of the treatment, Valox has very low porosity, making this equipment technique not practical and unfeasible at a real scale application.

The remaining issue to manage for scaling up the process is the larvae density to be used in a treatment process unit, in order to achieve the best treatment performance. Only in the study of Grossule et al. (2022), the treatment process was evaluated by using four different number of larvae to be placed inside the reactors. In the experiment each leachate was tested at four different loads by feeding with the same volume 20, 40, 80 and 160 larvae. However, since that research was not aimed at understanding the perfect larvae density, it was not possible to establish the influence of the number of larvae inside the reactors and the optimal larvae density to be used.

1.4 Research Objectives

The present study is aimed at filling the missing steps from the patent to the real scale application: 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.

2 MATERIALS AND METHODS

2.1 Research program

The experiment was performed by placing young BSF larvae (6 days old, 16,8 mg average wet weight per larva) 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^2) were tested with the same artificial wastewater in the different supports, under same organic load. The artificial wastewater was obtained by diluting low fat milk with water (1:10). The Total organic carbon (TOC), Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of the artificial wastewater was 2740 mgC/mL, 13800 mgO_2/L and 15300 mgO_2/L respectively. The Figure 7 depicts the configuration of the experiment.

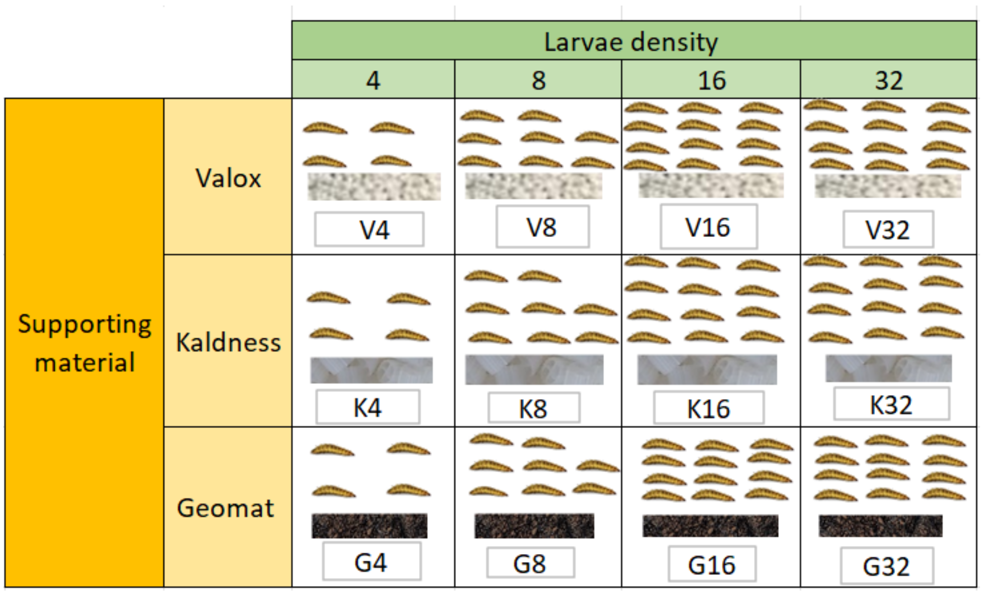

Figure 7: Graphical description of the experimental set up.

2.2 Equipment and operation conditions

Each testing reactor was made of plastic box (13.5 x 13.4 x 5.5. cm for 4, 8, and 16 larvae/cm^2, 13.5 x 13.5 x 16 cm for 32 larvae/cm^2), containing Valox® plastic granules, Kaldnes ®, plastic porous 3d structures, and geomat, drainage nets, completely saturated with artificial wastewater. Each box was covered by a permeable non-woven fabric (to avoid oviposition by other flies) and by a perforated plastic lid (to allow air recirculation). An example of the experimental set up of the reactors is provided by the Figure 8, displaying the larval density of 32 larvae/cm^2 tested with the three supporting materials. All tests were carried out in a thermal insulated room under the same environmental conditions suggested by Grossule et al. (2020): temperature range 25-30°C; photoperiod Light/Dark of 18/6h. The experiment was stopped at the 35th day when prepupation phase started.

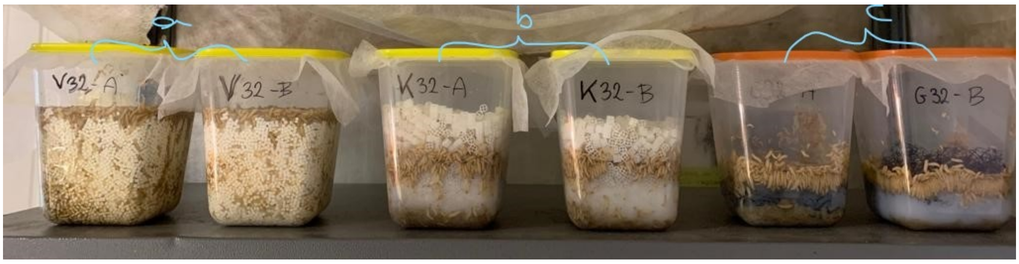

Figure 8: Experimental set up of part of the research a) Valox with 32 larvae/cm^2; b) Kaldnes with 32 larvae/cm^2; c) Geomat with 32 larvae/cm^2.

2.3 Feeding and monitoring

Each larvae density box was filled with specific volumes of artificial wastewater in order to guarantee a constant load and thus to provide the larvae the same amount of feed. Otherwise, without a constant load, it couldn’t be possible to discern if the differences of larvae growth were due to the larvae density or food shortages. The amount of load chosen was of 7,5 mL/d/larva/cm^2 distributed during the week over 5 days; in particular boxes containing 4 larvae/cm^2 were fed every day with 42 mL of artificial wastewater, with 84 mL, 168 mL, and 336 mL boxes accommodating 8, 16 and 32 larvae/cm^2 respectively. All the configurations were performed in triplicate. Every day for 5 days at the time of feed replacement, wastewater volume was sampled, measured, frozen and once a week analysed for TOC concentrations. Larvae were collected twice a week, washed, individually weighed using an analytical balance and returned to the box. Prepupae, recognised by darkening of their colour, were removed in correspondence to the weighing, they were washed, weighed and frozen. Larvae development and substrate consumption were monitored by measuring the following parameters: average larval wet weight variation, prepupation (% of prepupae over the total number of larvae), mortality (% of dead larvae over the total number of larvae), yield (mglarva/mgTOC) and specific substrate consumption rate (mgC/larva/d). Yield (Y) expresses the conversion efficiency of substate into new biomass by anabolic processes, it was calculated as follow:

$$Y= \Delta X/\Delta TOC =(mglarva/mgTOC) \quad \text{Eq.1}$$

ΔX= (final larvae weight - initial larvae weight); ΔTOC= sum of weekly substrate consumption.

The specific substrate consumption rate (v_s) was calculated according to the following equation:

$$V_s= S-S_0/X_0(t-t_0) = (mgC/larva/d) \quad \text{Eq.2}$$

$S-S_0$= removed substrate (mgTOC); X_0= initial number of larvae; $t-t_0$= monitoring time.

The Table 3 summarizes the operational conditions and the feeding programme.

larvae density (larvae/cm^2)	4	8	16	32
Temperature (°C)	25-30			
Photoperiod L:D (h)	16:08			
Duration (d)	35			
nr larvae / box	576	1152	2304	4608
Feeding/box/day (mL)	42	84	168	336

Table 3: Operating conditions and feeding of the four larvae density.

2.4 Analytical procedures

Artificial wastewater was analysed for the following parameters: TOC, BOD_5 and COD.

TOC was analysed by using a TOC-VCSN Shimadzu Analyzer; COD and BOD_5 were determined according to the standard Italian method IRSA-CNR (29/2003 vol 2 n. 5130; 29/2003 vol 2 n. 5120 B2).

3 RESULTS AND DISCUSSION

3.1 Larval growth

Figure 9, Figure 10 and Figure 11 illustrate the variation of larval growth (average larval wet weight, w/w) under four larvae density and different supporting materials tested over the testing period of 35 days. In the three different supporting materials, the average larval wet weight curves of 4, 8 and 16 larvae/cm^2 showed similarities in the growth development of larvae, the only exception was represented by the test V16 (Valox, 16 larvae/cm^2) in which a faster growth was obtained in the last 10 days of test. In K4, K8 and K16 (Kaldnes, 4, 8 and 16 larvae/cm^2) tests, analogous average larval wet weight values were achieved at the 35th day of 50,1 mg, 50,2 mg, and 52,8 mg (w/w) respectively. Also, in the case of Geomat reactors, the final average larval wet weight was similar in the three larvae density of 4, 8 and 16. A similar trend to V16 was exhibited by the 32 larvae/cm^2 tests for all the supporting materials; after 18 days of tests, the larval growth increased exponentially compared to the lower larvae density. This behaviour can be explained by integrating the larval growth development graphs with the specific daily substrate consumption values and the mortality rate of the larvae. In fact, even if larvae of 32 larvae/cm^2 tests and V16 became bigger than the ones of other tests, the specific substrate consumption (v_{TOC}, mgC/larva/d) was lower instead of being equal or like the other tests. This result is justified by the large number of dead larvae observed in 32 larvae/cm^2 reactor tests and in V16, which has led to higher organic load per larvae per day. This behaviour was the most evident in V16 and V32 tests in which mortality (% dead larvae over total initial number of larvae) reached the highest values of 74,82 and 75,27 % respectively. In conclusion at same organic load, larvae density from 4 to 16 showed similar growth variation, demonstrating that no competition between larvae occurred, while regarding the support materials small differences in the final average larval wet weight was recorded but it can be considered insignificant.

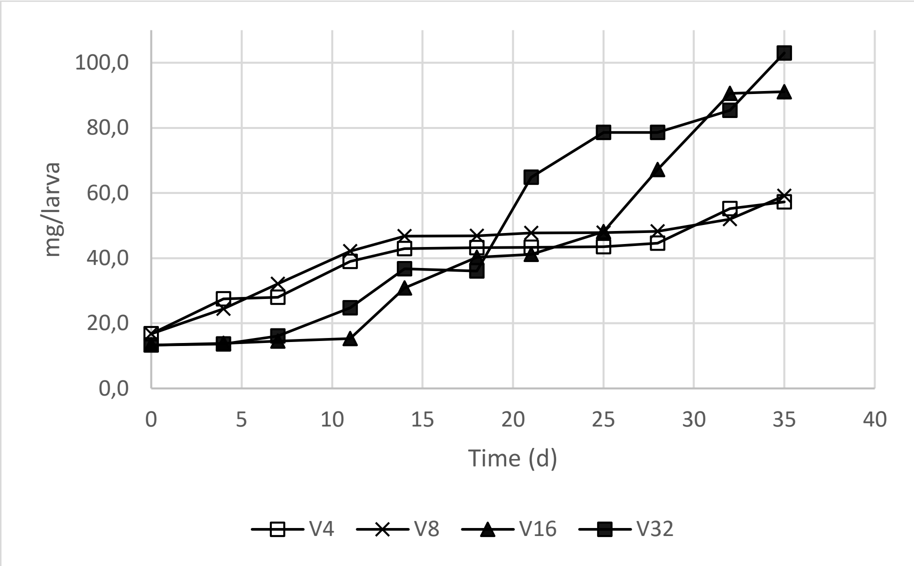

Figure 9: Average larval wet weight of Valox tests over time (V=Valox; 4, 8, 16 and 32 larvae/cm²).

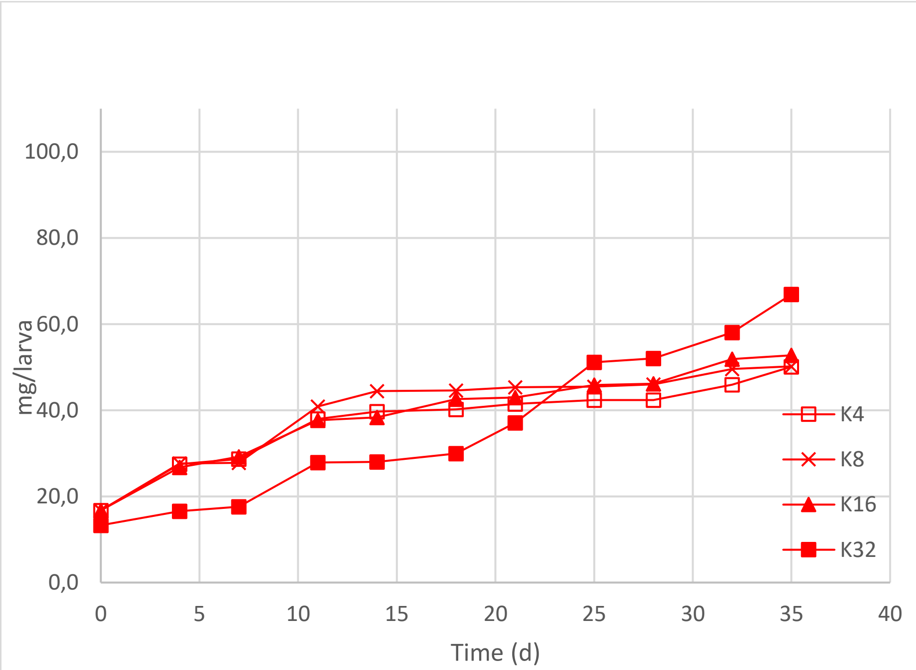

Figure 10: Average larval wet weight of Kaldnes tests over time (K= Kaldnes; 4, 8, 16 and 32 larvae/cm²).

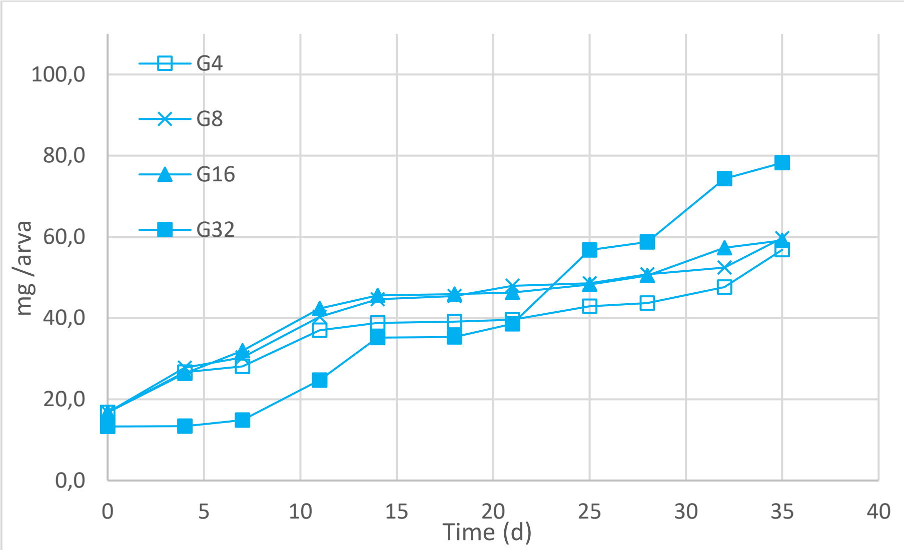

Figure 11: Average larval wet weight of Geomat tests over time (G=Geomat; 4, 8, 16 and 32 larvae/cm²).

During the experiment, the larvae that became prepupae were counted and removed, the first ones were observed at the 11th day in few reactors (V4, V8, K8, K16, and G8) and, at the 35th day when prepupation phase had begun through all the tests, the experiment was stopped. Figure 12 compares the prepupation percentages of four larvae density of each supporting material: the 4 larvae/cm^2 tests present similar values in the three supporting materials with the highest value of 6,83% and lowest value of 5,67% for Valox and Geomat respectively. On the contrary in 8, 16 and 32 larvae/cm^2 tests the difference between the materials was much greater with K8, K16 and V32 reaching the highest values respectively of 6,89%, 4,12 % and 2,01% while the lowest values were achieved by G8 with 2,78%, by V16 with 0,78% and by K32 with 0,2%. From these results it may seems that prepupation is influenced both by larvae density and by the solid support: generally with the increase of larvae density the prepupation % decreases.

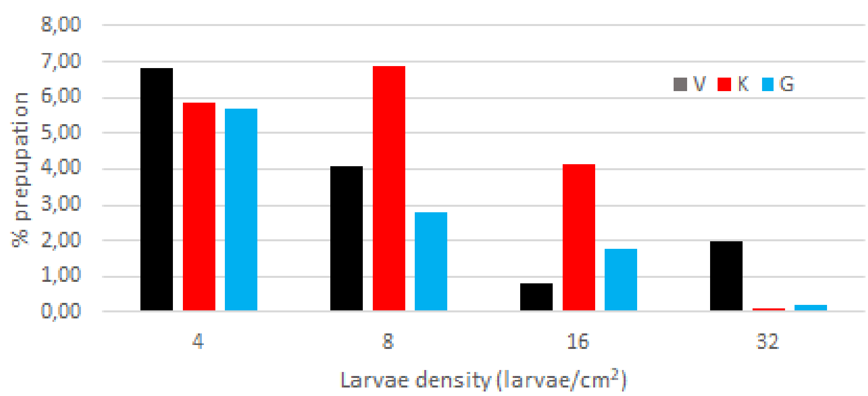

Figure 12: Prepupation (% prepupae over total number of larvae at the start; V=Valox; K=Kaldnes; G=Geomat)

As previously reported the highest mortality values were recorded in V16 and V32 boxes even if the solid support offered by the plastic granules were the greatest in terms of surface area and porosity. In fact, V4 test proved to be the best material of among the 4 larvae density tests with 19,3% of dead larvae. V8 test showed a slightly higher percentage of 21,6% compared to V4. On the contrary in Geomat tests, where the solid support was lower (in terms of surface area and porosity), the mortality values decreased from 4 to 16 larvae/cm^2 tests ranging between 25,5 % and 30,9 % (in G16 and G4 respectively), while for larvae density of 32 (larvae/cm^2) the percentage reached ca. 60%. Also, Kaldnes tests displayed a decreasing trend of mortality from K4 to K16 with the exception of K32, where it reached 47,7%. The 16 larvae/cm^2 seemed to be the limit number of larvae per quadratic centimetre for their survival, after that density the mortality grows till values of 60 %. The high mortality obtained in V16 can be ascribed by the little space left over for the respiration of larvae, a better configuration of the test was needed in order to provide the right conditions to larvae. In addition, it can be concluded that Kaldnes material provided the best solid support in terms of low mortality reached in all the larvae density apart from the 4 larvae/cm^2 in which Valox leads with 1 point %.

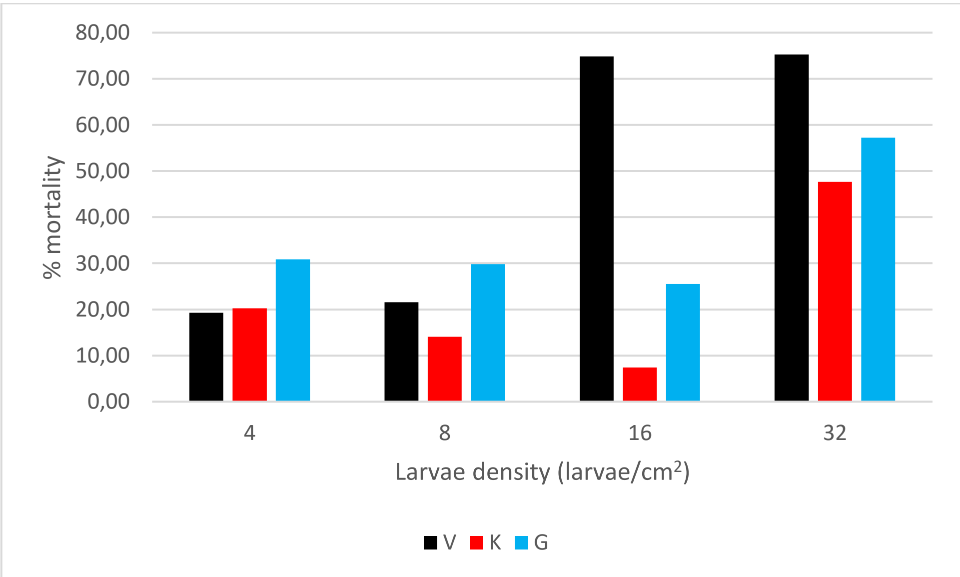

Figure 13 :Mortality (% dead larvae over the total initial number of larvae; V=Valox; K=Kaldnes; G=Geomat).

The mortality values confirmed the results obtained for the conversion of substrate into new biomass, expressed by the Yield (mglarva/mgTOC) parameter, graphically represented by Figure 14. In general, an increasing yield is observed from 4 to 16 larvae/cm^2 through all the tests except for V16 test. Geomat tests resulted in a lower conversion of substrate in respect to Kaldnes, while compared to Valox the lower values were detected just for the 4 and 8 larvae density. After the maximum yield value of 5,7 mglarva/mgTOC for G16 the conversion decreased reaching 4,6 mglarva/mgTOC for G32 test. Valox tests followed the same pattern as geomat and Kaldnes, in fact an increase from 4 to 8 larvae/cm^2 was detected; the amount of new biomass embodied dropped till a value of 2,5 mglarva/mgTOC in V16 due to the higher organic load per larvae, obtained as a consequence of the great mortality. The overall maximum yield value was achieved by K16 with a yield of 6,7 mglarva/mgTOC, after the peak value the yield of K32 decreased achieving 4,9 mglarva/mgTOC. In conclusion at same organic load, the greater the larvae density the higher the conversion efficiency of substrate into new biomass was achieved, but beyond 16 larvae/cm^2 an opposite effect was detected. Indeed, also in this case the low yield value was due to the high number of dead larvae present that led to higher load per larva; this amount of organic load was used by larvae for the catabolism activities instead of the anabolism processes, which are responsible for the conversion of organics into new biomass.

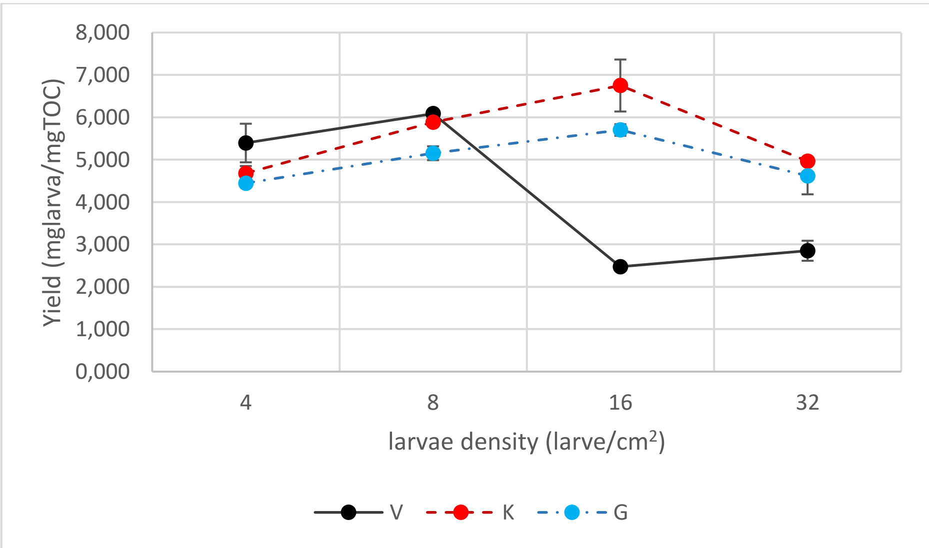

Figure 14: Yield under different larvae density and solid support (mglarva/mgTOC; V=Valox; K=Kaldnes; G=geomat).

3.2 Specific substrate consumption rate

Figure 15, Figure 16, Figure 17 represent the specific daily substrate consumption of the different material under four larvae density over the testing period, useful for designing a BSF larvae reactor. This parameter was calculated on weekly basis in terms of TOC consumption. Generally, v_{TOC} grew in line with the development of the larvae, meaning that substrate consumption increased with aging of larvae during the growth phase. The v_{TOC} curves followed a stiffer increase in the first 14 days then achieved a stabilized consumption value around ca. 0,140 mgC/larva/d, suggesting that from the 14^{th} day of test larvae have grown enough to be able to “eat” more substrate. The three different support materials didn’t display any substantial difference in the substrate consumption, apart from V16 which differed from the others 16 larvae/cm^2 tests. At the end of the experiment BSF larvae of the four larvae density presented more or less same values of specific substrate consumption ranging ca. between 0,137 and 0,140 mgTOC/larva/d for 4,8 and 16 larvae/cm^2 tests and between 0,131 and 0,134 mgTOC/larva/d for 32 larvae/cm^2 tests. Confirming that v_{TOC} values increase linearly as the organic loads increase (Grossule et al., 2022), thus if the organic load is constant also the consumption rate across the four larvae density is similar or equal. The differences between 32 larvae/cm^2 and the other larvae density are consistent with the results obtained in the mortality and in the conversion efficiency of substrate into new biomass. In fact, the lower consumption values obtained in V16 test and 32 larvae/cm^2 are justified by the large number of dead larvae: as the v_{TOC} is calculated from the ratio between the TOC consumed and number of larvae initial over one week, if the larvae alive are few, the organic carbon consumed is less and therefore also the specific substate consumption. All the boxes from 4 to 16 larvae/cm^2 of the three solid supports showed to be indifferent form the point of view of specific substrate consumption.

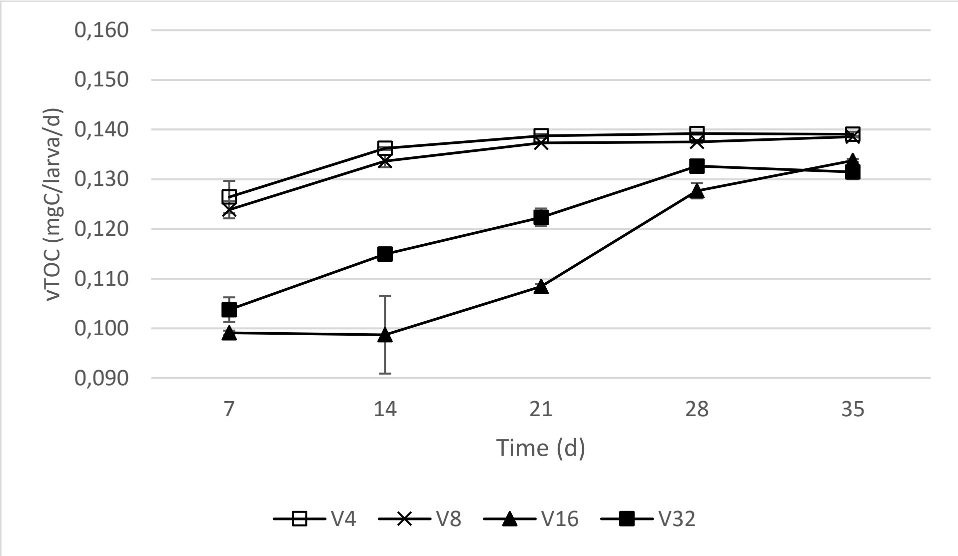

Figure 15: Specific consumption rate over time (mgTOC/larva/d; V=Valox; 4, 8, 16 and 32 larvae/cm²).

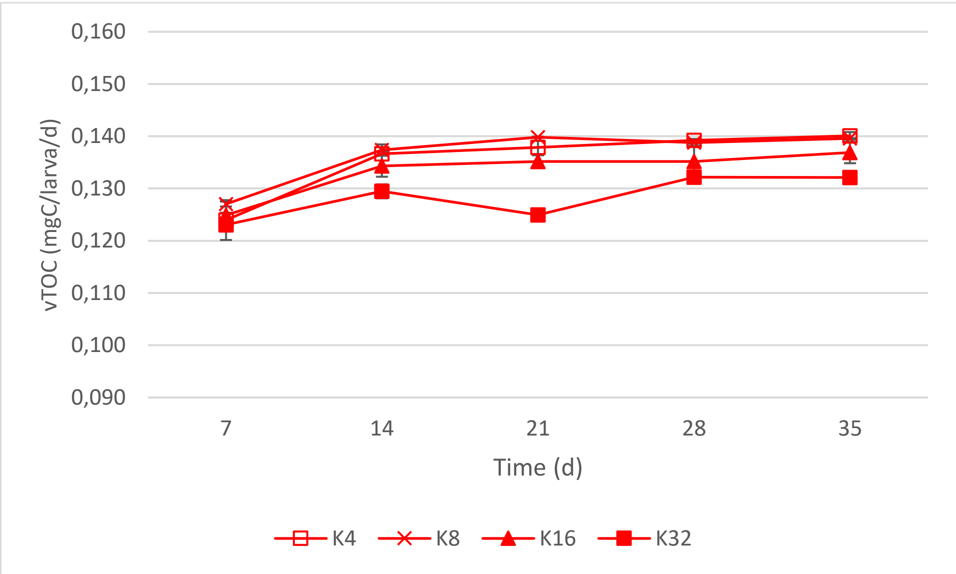

Figure 16: Specific consumption rate over time (mgTOC/larva/d; K=Kaldness; 4, 8, 16 and 32 larve/cm²).

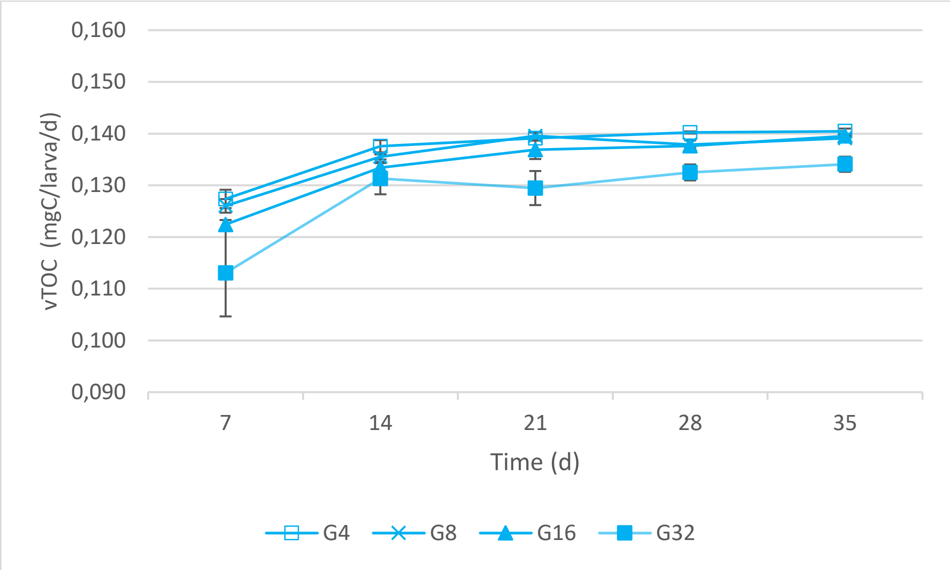

Figure 17: Specific consumption rate over time (mgTOC/larva/d; G=geomat; 4, 8, 16 and 32 larvae/cm²).

4 CONCLUSIONS

The influence of larvae density and solid support on wastewater treatment performance using the BSF larvae was investigated by monitoring both larvae growth (in terms of weight variation, mortality and prepupation), and wastewater quality and quantity variation to determine the substrate consumption. Based on the results obtained, excluding the test with valox and 16 larvae/cm^2 being affected by technical problems, the following conclusions can be drawn:

- Larval growth, in terms of overall larval wet weight, mortality prepupation and yield, was significantly negatively influenced by high larvae density (32larvae/cm^2), 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^2 regardless the solid support. The only exception was test with Valox material and 16 larvae/cm^2 where variation of average larval wet weight was faster and greater, similarly to all tests with larvae density of 32 larvae/cm^2. 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^2; differences between yield values of supporting materials were detected with the maximum value obtained by K16 test.
- The specific substrate consumption rate (v_s) was consistent with the other results, displaying lower values in correspondence of 32 larvae/cm^2. v_s values were not influenced by the supporting materials used.

As final conclusion, the optimal operating conditions for real scale wastewater treatment using BSF larvae include the use of Kaldnes as supporting material and of 16 larvae/cm^2 as larval density, being the optimum trade of between the need of maximise the larval density and the effective reactor volumes without compromising larvae survival.

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