PROJECT FINAL REPORT

Grant Agreement number: 311956
Project acronym: SPLASH
Project title: Sustainable polymers from algae sugars and hydrocarbons
Funding Scheme: 12, 18, 24 month
Period covered: from 1.8.2013 to 28.2.2017
Name of the scientific representative of the project's co-ordinator[1], Title and Organisation:
Dr. Lolke Sijtsma, stichting Wageningen Research (previously: Dienst Landbouwkundig Onderzoek)
Bornse weilanden 9, 6708 WG, Wageningen, The Netherlands
Tel: +31 (0)317 480220
E-mail: lolke.sijtsma@wur.nl
Project website Error! Bookmark not defined. **address: www.eu-splash.eu**

[1] Usually the contact person of the coordinator as specified in Art. 8.1. of the Grant Agreement.

4.1 Final publishable summary report

Executive summary

The SPLASH project aimed to develop a new biobased industrial platform using microalgae as a renewable raw material for the sustainable production of polymers. Microalgae are a promising new renewable feedstock for e.g. chemicals and plastics. They can be cultivated on non-arable land and can yield valuable compounds for chemical industries. If microalgae can be sustainably cultivated at an industrial scale this will provide new opportunities for decreased dependency on fossil feedstocks, and potentially contribute to climate mitigation and reduced pressure on land resources.

In our approach we used the microalga *Botryococcus braunii* for the production and recovery of hydrocarbons and (exo)polysaccharides and their further conversion to renewable polymers such as bioplastics. Furthermore, the model microalga *Chlamydomonas reinhardtii* is included as potential production host to test unique hydrocarbon and polysaccharide-producing genes from *B. braunii*.

The project encompasses (1) development of *B. braunii* as an industrial production platform, (2) systems biology analysis, (3) development of procedures for production, in situ extraction and isolation of hydrocarbons and carbohydrates, (4) product development and (5) costs and sustainablilty analysis

After 4,5 years of research we developed tools and technologies needed for the establishment of a new industrial sector: Industrial Biotechnology with algae and/or algal genes for the manufacture of industrial raw materials. We built a foundation for commercialisation of hydrocarbon production with the *B. braunii*. We developed knowledge to understand the product formation of two *B. braunii* strains that produce mainly hydrocarbons or polysaccharides respectively, based on in-depth gene analysis. We improved algae production and were able to recover hydrocarbons and polysaccharides while keeping the algae viable, although further improvements are required. Furthermore, we made substantial progress in the analytics of carbohydrate and hydrocarbon fractions and the conversion of carbohydrates into building blocks and subsequently polymers such as fibres for yarns. As industry, however, needs relatively cheap and pure raw materials, costs for sugars produced by *Botryococcus braunii* are currently far too high. Furthermore, separation of sugars from mixtures remains an important item to be addressed. We developed a pilot facility capable of demonstrating an optimized process for the production and utilisation of both polysaccharides and hydrocarbons and identified potential business cases for *B. braunii* hydrocarbons based on realistic assumptions. Public and potential stakeholders were informed about the outcome and benefits of the project. Conferences, meetings and a summer school were organised in order to inform and train researchers (academic and industrial) in the fields of metabolic modeling and engineering, bioconversion, microalgae cultivation, downstream processing, conversion of biomass (especially hydrocarbons and polysaccharides into chemicals), life cycle assessment and process modeling.

Summary description of project context and objectives

SPLASH: Sustainable polymers from algae sugars and hydrocarbons.

Project context and objectives
Around the world steps are being taken to move from today's fossil based economy to a more sustainable economy based on biomass. Microalgae may be a promising new renewable feedstock for e.g. chemicals and plastics. They can be cultivated on non-arable land and can yield valuable compounds for chemical industries. If microalgae can be sustainably cultivated at an industrial scale this will provide new opportunities for decreased dependency on fossil feedstocks, and potentially contribute to climate mitigation and reduced pressure on land resources (Brochure SPLASH 2017).

The aim of the 4.5-year SPLASH project was to develop a new biobased industrial platform using microalgae as a renewable raw material for the sustainable production and recovery of hydrocarbons and (exo)polysaccharides from the species *Botryococcus braunii* and further conversion to renewable polymers. Main focus points were:

- Understanding of product formation of two *B. braunii* strains that produce mainly hydrocarbons or polysaccharides respectively, based on in-depth gene analysis
- Development of understanding and procedures for production, in situ extraction and isolation of hydrocarbons and carbohydrates from selected *B. braunii* strains for further product development
- Conversion of hydrocarbons or polysaccharides to products
- Process demonstration at pilot scale, Process integration, sustainability assessment and market analysis

By using an integrated chain approach – ranging from systems biology to product development – and including economic and environmental impact, the feasibilities and challenges for future industrial implementation can be better addressed. In the sections below the main focus points have been described in more detail

1. Understanding of product formation of two B. braunii strains based on in-depth gene analysis

The main thrust of this work has been to use genomics and systems biology to understand the production of hydrocarbon and (exo)polysaccharide production in *Botryococcus braunii* at the fundamental level. This will underpin efforts to develop metabolic engineering strategies to generate industrial host strains, but also provide leads for cultivation concepts, improved growth and specific product enhancement to be exploited.

To reach the objectives two *B. braunii* strains with desired properties were selected from a set of 16 obtained from culture collections. Strain CCALA-778, a race A strain, produced carbohydrates to over 50% of its cell dry weight, but did not contain measurable levels of hydrocarbons under our experimental conditions, whereas strain AC-761, a race B strain, produced around 40% (w/w) of hydrocarbons, and contained some carbohydrates.

In our approach, the selected *B. braunii* strains were cultivated in controlled air-lift bioreactors, and biomass samples produced for analysis of genome sequence and their intracellular metabolism using transcriptomics, proteomics, and metabolomics technologies . An initial systems model of *B. braunii* growth and product formation was generated using published information from the literature and databases. This has been refined using data generated in the SPLASH project. In parallel, molecular tools for metabolic engineering of the model green alga *Chlamydomonas reinhardtii* were developed and these have been used to start to verify the function of *B. braunii* genes. Although no mutants could be recovered via mutagenesis treatment as since single *B. braunii* cells are not viable, UV treatment of CCALA778 substantially reduced the bacterial load.

The presence of bacteria in the *B. braunii* colonies was identified early on as a key bottleneck in activities regarding *in-depth gene analysis*. For one, the large bacterial community hampered extraction of DNA and RNA, and then most of the sequence that was obtained was from bacterial sources. Initially short-read Illumina sequencing technology was used, and the assembly of genomic DNA was essentially the metagenome. Moreover, bacterial and algal contaminations became a problem for e.g large scale cultivation systems which demonstrated highly variable results depending on the extent of the contamination.

For *in-depth gene analysis* difficulties were tackled in several approaches, and most have been overcome. Firstly, PacBio sequencing, which yields much longer sequences, provided data of sufficient quality to allow algal genome sequences to be assembled. At the same time genomes of several of the associated bacteria have been assembled, and this will allow a systems level analysis of the function of the whole consortium. In addition, a Race A strain, SAG-30.81, was identified that had a much lower bacterial content. As well as providing much cleaner DNA for sequencing, it has also enabled us to establish that, while the presence of bacteria may enhance growth of *B. braunii* (SAG 30.81 grows even slower than other strains) it is not necessary for hydrocarbon or polysaccharide production. This conclusion is supported by studies on the CCALA 778 strain after UV treatment.

2. Procedures for production optimization, in situ extraction and isolation of hydrocarbons and carbohydrates from selected B. braunii strains for further product development

Media development for the sugar polymer producing *B. braunii* CCALA-778 resulted in 2-fold biomass productivity and 3-fold exopolysaccharides productivity compared to reference media. Selection of proper commercial fertilizers, on basis of developed knowledge, also yielded good productivities whereas media price could be successfully reduced.

Process optimization showed that day-night cycles increase biomass and exopolysaccharide productivity. Optimal temperature is 26ºC although *B.braunii* can grow in a range from 16ºC to 31ºC. According to our results, it is possible to cultivate *B.braunii* CCALA778 in a continuous way in the Mediterranean basin along the year. However, it is important to have an accurate control of the temperature within the range from 20 to 30ºC. Polysaccharides extraction ("milking") could be performed with a relatively straight forward approach by a combination of cell retention by 0.2 µm Microfiltration and concentrating the permeate by a 10 kDa Ultrafiltration step. By doing so a more or less sterile EPS concentrate is obtained which can be further processed.

For the hydrocarbon (EHC) producer *B. braunii* AC761, selenium could be removed successfully from the medium without loss of productivity which is an important achievement in terms of safety and environmental protection. Also for this strain the usage of commercial fertilizers reduced medium costs.

A model, developed for hydrocarbon production showed light intensity has a positive influence on hydrocarbons productivity. Hydrocarbons (figure 1.) can be extracted by organic solvents

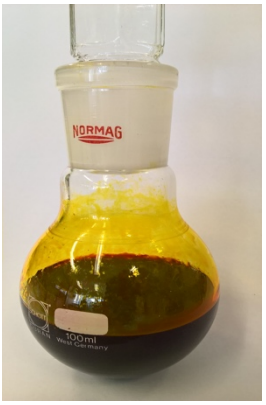


Figure 1: Hydrocarbons extracted from *Botryococcus* Showa (race B)

Extraction of hydrocarbons from cultivation medium and biomass by "milking" *B.braunii* is possible at laboratory scale by trickling the algae broth through an organic solvent layer. The EHC can be further concentrated and the solvent recovered by vacuum distillation. Care, however, needs to be taken in order to keep the algae alive and producing the product of interest. In a process with low damage to the algal cells, the best extraction result was about 40% of hydrocarbon recovery. Although the Sonic Fluid Flow Process could be applied to wet biomass, this process caused algal death and was not applicable for the continuous extraction ("milking") process of EHC

3. Conversion of hydrocarbons or polysaccharides to products

In order to develop building blocks and subsequently products from algae exopolysaccharides (EPS) and exohydrocarbons (EHC) this part of the research concerned (i) the conversion of EHC into ethylene and propylene by way of catalytic cracking and (ii) hydrolysis of EPS to monosaccharides and conversion of monomers for production of polyesters and polyamides: adipic acid, 2,5-furandicarboxylic acid (FDCA) and 1,4-pentanediol.

The approach of Fraunhofer for EHC cracking was to use steam cracking directly on dissolved botryococcenes. According to the experiences from petrochemical refining, applying steam cracking directly should maximise the share of the light olefins were the SPLASH project aims for. The actual feedstock was planned to be botryococcenes blended in hexadecane. With hexadecane as model feedstock, ethylene and propylene yields as high as 20% and 11%, respectively, were obtained. The results show that the bench-scale

plant, implementing the one-step pathway of steam-cracking, is suitable for cracking larger hydrocarbons into a gas phase rich in ethylene and propylene. However, the unexpectedly high nitrogen content of 0.56 wt% found in EHC makes an industrial route from EHC to ethylene and propylene as platform chemicals unrealistic. It is favourable to focus on extracting the valuable chemicals of *Botryococcus* hydrocarbons, purifying them and utilising them without or with only small modifications in the molecule structure.

EPS is a long chain polymer and due to its pseudo-plastic behaviour it has potential to be applied in e.g. wall paints as such. The main EPS sugars were galactose and fucose. One of the objectives was the conversion of monosaccharides, derived from the EPS, into di-acids and diols. Due to limitations in availability and purity of algal derived sugars, commercially sugars were used to demonstrate the processes. Galactose can be converted into adipic acid in a multistep synthesis. The overall yields were, however, always significantly below 50 percent.

The relative amounts of fucose and rhamnose in EPS can vary, depending on the type of *B. braunii* strain used. Since both sugars have the same chemical structure and only vary in the stereochemical configuration, the cheapest and most readily available commercial carbohydrate of the two: rhamnose was uses. 1,4-pentanediol was successfully synthesised in multiple steps, although the overall yield must be significantly improved.

The conversion of C6 sugars into furans is well known. In recent years, Avantium has developed the YXY-process which starts from sugars to produce 2,5-furandicarboxylic acid (FDCA), which can be polymerised to polyethylene furanoate (PEF). In October of 2016 Avantium and BASF formed a joint venture to commercialise the production of PEF. For effective dehydration to furans, it is favourable to use ketohexoses as feedstock. It was discovered that psicose was the most favourable feedstock for the production for alkoxymethylfurans. This finding is patented. Due to the small amount and purity of available *B. braunii* carbohydrates in the earlier stages of the project, no experiments have been performed with sugars obtained directly from algae.

Polymer production: The polyester of 1,4-pentanediol and adipic acid was prepared for the first time. and resulted in a sticky polymer. In addition, other biobased building blocks were tested in combination with adipic acid and 1,4-PDO, resulting in different material properties. PEF was successfully used for fibre production (Figure 2), with good spinability, potential for stretching and a resulting fibre with good mechanical properties and acceptable abrasion resistance. The results show PEF as a good candidate for industrial textile applications.

Figure 2: Fibres prepared by Lankhorst Euronete from PEF provide by Avantium

4. Process demonstration at pilot scale, Process integration, sustainability assessment and market analysis

Microalgae-derived biobased products (i.e., biofuels, biochemicals and biopolymers) have received increasing attention as one of the solutions to the continued and growing dependency on fossil resources. In this context, *B. braunii* offers interesting opportunities as it secretes unique extracellular polymers. In order to evaluate the economic feasibility and sustainability In these deliverables, a mathematical model for the microalgae-based production of extracellular polysaccharides (EPS) and hydrocarbons (EHC) and their conversion to respective biopolymers was developed. Eight scenarios were selected, simulated and evaluated with the developed model.

It was found that the major hotspot for both capital and operation expenditures is the cultivation of microalgae in the photo-bioreactors, followed by the milking/separation step. From the selected key-process

parameters, the biomass specific growth rate and the EPS/EHC yield are the ones with the major effect on the production cost, followed by the milking and drying/separation efficiency.

Despite these hotspots, scenarios that consider EPS and EHC as the final products are the most promising ones (Figure 3, Optimised Sales Price is within the Market Price Range and have enough sales arguments to be commercially attractive), because of more simple plants and higher values of the end products. The remaining four scenarios (S3a, S3b, S3c and S4), where the EPS and EHC are converted to (building blocks for) biopolymers, seem not viable under the current conditions. This is due to the more complex processes and the low value end products.

The environmental impacts were determined by life cycle assessment (LCA). The impacts of dry EPS and dry EHC are dominated by the energy use of isolating and, especially, drying the EPS and EHC. The SPLASH adipic acid and, to a lesser extent, FDCA production pathways can be considered environmentally competitive, especially when using renewable energy for all power and heat needs of the SPLASH developed production steps. Without additional process improvements, SPLASH 1,4-pentanediol, ethylene, and propylene are unable to compete against their mass-produced competition on the level of 'environmental impact'.

	Product	Proposed volume	Proposed sales price	Optimised sales price	Market price range	GO/ NO GO
S1a	EPS	20.000 T	€6/kg	**€5/kg**	€7-15/kg	
S1b	EPS	200 T	€15/kg	**€43/kg**	€15-20/kg	
S2a	EHC	10.000 T	€4/kg	**€6/kg**	€4-6/kg	
S2b	EHC	500 T	€20/kg	**€20/kg**	€30-45/kg	
S3a	1,4-PDO	2.000 T	€5/kg	**€17/kg**	€2-5/kg	
S3b	2,5-FDCA	10.000 T	€3/kg	**€11/kg**	€1/kg	
S3c	Adipic acid	10.000 T	2,5/kg	**€9/kg**	€1,5-2,5/kg	
S4	Ethylene	100.000 T	€1,5/kg	**€4/kg**	€0,5-1,1/kg	
S4	Propylene	100.000 T	€1,5/kg	**€4/kg**	€0,75-1,3/kg	

Figure 3. Cost estimation of *B.braunii* products

EPS ExoPolySaccharide;
EHC ExoHydroCarbon;
PDO Pentane DiOl
FDCA FuranDiCarboxylic Acid

It can be concluded that the in SPLASH developed cultivation and milking technologies have the potential to operate economically, in particular for EHC, provided that all the involved processes will be optimized and the targeted products are of higher than commodities value.

Public and potential stakeholders were informed about the outcome and benefits of the project. The website (www.eu-SPLASH.eu) and social networks have regularly been updated and three press releases were sent to a broad distribution lists. Furthermore the project co-organised and strongly participated in the organization of the International Conference "European Roadmap for an Algae-Based Industry" held in Olhão (Portugal) in April 2016. The Summer School "Microalgae Biorefinery" in Wageningen (The Netherlands) in July 2016 was organised in cooperation with Wageningen University in order to inform and train researchers (academic and industrial) in the fields of metabolic modeling and engineering, bioconversion, microalgae cultivation, downstream processing, conversion of biomass (especially hydrocarbons and polysaccharides into chemicals), life cycle assessment and process modeling.

Partnership

In order to achieve the overall aim and the specific objectives the consortium consisted of a multidisciplinary team including partners who are leading in their work field. The consortium consisted of a mix of established research organisations and universities, small and medium enterprises (>50% of total partners) and large scale industry. These are: Wageningen Food & Biobased Research, Wageningen Plant Research, Wageningen University, Centre for Research and Technology, Organic Waste Systems nv, Paques bv, Norsker Investigationes, Value For Technology bvba, Avantium chemicals bv, Lifeglimmer gmbh, Nova-institut fur politische und okologische innovation Gmbh, Fraunhofer-Gesellschaft zur Förderung derAngewandten Forschung e.v, University of Cambridge, PNO consultants bv, Universidad de Huelva, Universitaet Bielefeld Westfaelische Wilhelms-Universitaet Muenster, Ege universitesi, Lankhorst euronete Portugal sa, Solvay (Rhodia), Cellulac, Fotosintetica & Microbiologia S.r.l.

Description of the main S&T results/foregrounds

WP2. From systems biology to strain engineering

Overall objectives of WP2
Use genomics and systems biology to develop metabolic engineering strategies for hydrocarbon and (exo)polysaccharide production in green algae, thus providing leads for cultivation concepts, improved growth and specific product enhancement.

Key findings and conclusions
2.1 Based on nuclear sizes reported earlier by WUR-PRI and published papers, the genome size for *B. braunii* race A and race B genomes is assumed app. 166Mb. From the assembly statistics we conclude that the nuclear genome from CCALA778 and SAG30.81 have been established. Due to extensive bacterial contamination the genome for AC761 requires additional sequencing.

Full length transcriptome sequencing provided specific details on the intron - exon structure of genes and enables to discriminate between the bacterial and algal genomes. The mapped full lengths transcripts have subsequently been used for gene prediction and extraction of coding and protein sequences. In addition RNAseq transcriptome data from differential growth experiments have also been mapped to the genome to further support the differential expression analysis.

2.2 Changes in transcripts, proteins, and metabolite levels of two *B. braunii* races A and B grown in fully controlled photobio-reactor systems were measured. The main aim of this investigation was to link differences in metabolome and proteome profiles to differences in genetic background, transcripts, growth conditions and biomass production, providing thereby clear understanding of the cellular biochemical processes underlying hydrocarbon and polysaccharide biosynthesis.

Our data on the metabolic profiles of the two *Botryococcus braunii* strains suggests that the strains differ mainly in sugars, hydrocarbons, sterols and fatty acids they produce. Thereby is noticeable that Race A is mainly producing sugars (exopolysaccharides) and the Race B produces hydrocarbons. Based on comparative quantitative analyses we found significant differences e.g. in enzymes of sugar metabolism, MEP pathway and enzymes involved in botryococcenes production in races A versus B of *B. braunii*.

2.3 A genome scale metabolic model for the distantly related green alga *Chlamydomonas reinhardtii* , supplemented with data generated in the SPLASH project was used as the basis for constructing one for *B. braunii*. It was possible to generate the first systems model of *B. braunii*.

2.4 We are now able to predict the changes in metabolic flux necessary for the formation of hydrocarbons in Race B, and polysaccharides in Race A. This is the most detailed *in silico* representation of *B. braunii* in the field, and will be a valuable tool for analysing metabolic flux distribution of *B. braunii* and the engineering of any of its pathways.

2.5 Isolation of a mutant strain from the selected *B. braunii* SPLASH strains was unsuccessful. There are at least two reasons for this. First the presence of a large amount of bacteria in the cultures of *B. braunii* and second the difficulty of *B. braunii* to start growing from a single cell. UV treatment of the carbohydrate producing strain, however, appeared effective in reducing bacterial contamination.

2.6 Due to improvements in reporter gene expression (2.7) we applied our standardised workflow for transformation to the expression of *B. braunii* derived genes in *C. reinhardtii*. These included the small subunit of RuBisCO (RBCS), in a *C. reinhardtii* mutant lacking the gene, and the genes for the *B. braunii* pathway for botryococcene biosynthesis. The result demonstrates that unmodified *B. braunii* genes can be expressed directly in *C. reinhardtii*, but that there may be differences in chloroplast import machinery between the two species.

Botryococcenes are methylated triterpenes applicable in the biofuel industry. Their biosynthesis requires three genes, squalene synthase-like (SSL) 1, SSL3, and TMT, to convert farnesyl pyrophosphate, an intermediate of isoprenoid biosynthesis, to botryococcenes. Using the unmodified *B. braunii* genes, constructs were generated

for introduction into *C. reinhardtii* so that they were under the control of the PSAD promoter/5'UTR and CA1 3'UTR (identified in 2.7 as optimal) and were transformed into *C. reinhardtii*. Five strains tested positive for the presence of the TMT gene, of which two also carried the SSL1/3 gene. In two strains the expression of TMT was confirmed but none of the 5 strains expressed SSL. Further transformants are being generated.

Taken together, by employing a standardised workflow and applying the results obtained from the work relating to 2.7, we have been able to verify the identity and function of at least one candidate gene from *B. braunii* in *C. reinhardtii*. The platform will be a valuable resource for further analysis of *B. braunii* genes identified as important from the systems level analysis of the genome, transcriptome and proteome.

2.7 In order to advance *Chlamydomonas reinhardtii* as a biotechnology host, reliable and reproducible tools for genetic transformation and metabolic engineering are required. To achieve this we have adopted the principles of synthetic biology by employing the design-build-test-learn cycle adapted from conventional engineering. This system allowed us to quantitatively investigate the impact of specific variables on the results of transgene expression. Contrary to data commonly published in the field, which is based on the analysis of a small number of transformants, we have analysed several hundred independent transformants. We chose to look first at the impact 3' UTRs on recombinant protein production. Several plasmids were made from constituent DNA parts using isothermal Gibson assembly. The design included 3'UTRs that have been widely used in *C. reinhardtii* (RBCS2 and PSAD), as well as several others identified from RNAseq data. We demonstrated that the 3'UTR sequences derived from carbon anhydrase 1 (CA1) and nitrate reductase (NIT1) improved workflow efficiency and increased the number of transformants expressing the desired transgene.

At the same time, standardized workflows for the generation, identification and characterization of transformants, were established that facilitate the introduction of more than one gene into *C. reinhardtii* (necessary for metabolic engineering). Moreover, we have now adopted the standardized syntax for Golden Gate assembly of constructs, which allows parts (promoter, UTRs, introns, targeting peptides etc) cloned into Level 0 plasmids to be combined easily and in different permutations to generate single and multiple gene constructs for transformation. The data generated can be used to populate 'parts' libraries and to inform subsequent experiments designed to over express specific transgenes in *C. reinhardtii*

WP3 Develop innovative cultivation and downstream concepts for improved growth, product enhancement and integrated recovery of exopolysaccharides and hydrocarbons

Overall objectives of WP3
Develop innovative cultivation and downstream concepts for improved growth, product enhancement and integrated recovery of exopolysaccharides and hydrocarbons.

Key findings and conclusions
3.1 Media optimization for *B. braunii* CCALA-778 resulted in 2-fold biomass productivity and 3-fold exopolysaccharides productivity compared to reference media. For *B. braunii* AC761, selenium could be successfully removed from the media with similar biomass and hydrocarbons productivities. This is an important achievement in terms of safety and environmental protection.

3.2 In order to optimise outdoor process commercial fertilizers (Agralia Fertilizantes S.L. and GAT-Fertiliquidos S.A.) were evaluated for two *B. braunii* strains – CCALA-778 and Showa. Nitrate and urea showed the best results as N source and biomass productivities were similar. According to that results, 5 commercial fertilizers were finally selected and evaluated. A commercial solution of Micronutrients was also supplemented to the NPK fertilizers in order to avoid any micronutrient limitation. Media price could be successfully reduced while improving product productivity for both strains.

Optimization of polysaccharide production under Mediterranean climate conditions was performed by simulating of light and temperature variations found inside an outdoors tubular photo-bioreactor located in Huelva (Andalusia, Spain) during winter and summer conditions and comparing it with different temperature and light controlled conditions (performed indoor). Our results suggest that day: night cycles increase biomass and exopolysaccharide productivity. Optimal temperature is 26ºC although it is tolerant to a wider range (16 to

31ºC). High light intensities as well as a low nitrogen supply favour exopolysaccharide production. This was two times higher in summer than in winter when temperature was controlled.

For the hydrocarbon producing *Botryococcus braunii* a mathematical model is developed to optimize colony size, biomass and hydrocarbon productivity. The model for biomass productivity shows that the variable temperature has a positive effect in biomass productivity

These models are powerful tools that permit to change the culture conditions in order to increase biomass and hydrocarbon productivity as well as the elucidate the effect of those in the size of the colonies.

3.3 Biomass (produced in reactors with volumes ranging from 600 L – 5 m^3, Huelva Spain) as well as extracted sugar polymers and hydrocarbons were produced for process development and further processing. Biomass has been used within WP3 mainly for developing in situ Down Stream Processing (DSP) methods. This is the milking concept within EU-SPLASH: collecting polysaccharides (EPS) or long chain hydrocarbons (HC) while keeping the algae cells viable. The amounts of biomass and product produced were not sufficient to for performing large scale processing and polymer production.

3.4 Different downstream processing (DSP) techniques were tested to extract hydrocarbons or carbohydrates while keeping the cells alive: the milking concept within EU-SPLASH.

After milking viable cells should be recycled to the photo bioreactor (PBR) so they can produce more of the desired products. This has impact on the DSP techniques as they should be mild as well as robust to avoid scaling up problems later.

- Extraction of EPS by milking B.braunii is relatively straight forward by a combination of cell retention by 0.2 μm Microfiltration and concentrating the permeate by a 10 kDa Ultrafiltra-tion step. By doing so a more or less sterile EPS concentrate is obtained.
- Extraction of HC by milking *B.braunii* is possible by trickling the algae broth through a cyclohexane solvent layer. The HC can be further concentrated and the solvent recovered by vacuum distillation. Further optimization is needed.

Although oil was liberated, Supersonic fluid processing is currently not an appropriate technique for releasing hydro-carbons for milking of the *B. braunii* algae die to a loss of viability. Supercritical CO2 extraction is not an appropriate technique for extracting hydrocarbons when milking the *B. braunii* algae due to the loss of viability.

3.5 For a proof of concept for an industrial process of product recovery in a continuous and stable way we focused on exopolysaccharides produced by *B.braunii* CCALA778. The feasibility of a milking process relies on the efficiency of the extraction. According to our results, it is possible to milk CCALA778 but there is still a big gap for a 100% efficiency in the process. In order to extract 12% of the exopolysaccharides daily without compromising the viability of the cultures it was necessary to start the microfiltration always with clean modules. During summer conditions in Wageningen the biomass density remains the same (4g/L) as well as exopolysaccharide content (0.5g/L) day after day, extracting approximately 0.06 to 0.1g/L*d of exopolysaccharides.

3.6 The SPLASH management team has appointed a Biosafety officer (BSO) who has been in charge of implementing the Directive 2000/54/EC (-biological agents at work) and Directive 2009/41/EC (-on the contained use of genetically modified micro-organisms). The Biosafety officer got a mandate from the management team to give a binding advice on biosafety issues.

WP 4 Product development and testing

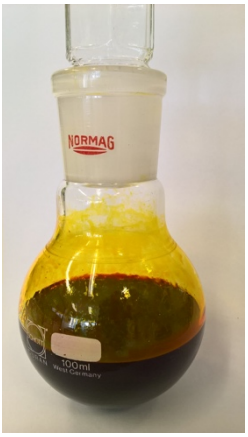
Overall objective of WP4

Develop processes to convert the hydrocarbons and polysaccharides into different monomers and polymers.

Research concerns both the conversion of exopolysaccharides (EPS) and exohydrocarbons (EHC). The EHC were intended to be converted into ethylene and propylene by way of catalytic cracking. The EPS were to be hydrolysed to their monosaccharides. These monosaccharides should subsequently be used as building blocks to synthesise monomers for polyesters and polyamides: adipic acid, 2,5-furandicarboxylic acid (FDCA) and 1,4-pentanediol.

Key findings and conclusions

4.1 Hydrocarbons were detected in 85 extracts. 36 samples were identified as race A, as mostly odd-numbered *n*-alkadienes and alkatrienes of chain lengths between C25 and C31 were detected. Fortynine samples were assigned to race B because of the large content of polymethylated unsaturated triterpenes (C30 to C37), called botryococcenes. The results were the basis for species selection and thus for hydrocarbon production in WP3.



One final hydrocarbon sample, originally produced to be further processed to ethylene and propylene in Task 4.2 and shown in Figure 1, was produced by extraction from *Botryococcus* Showa (race B) via solvent extraction.

Figure 1: Hydrocarbons-extract from *Botryococcus* Showa (race B)

In the sample 85 wt.-% hydrocarbon content was measured with structures comparable to squalene (Figure 2). The sample's ash content was < 0.001 wt.-%.

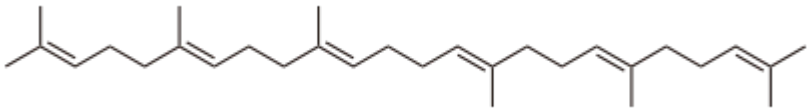


Figure 2: Chemical structure of squalene

Elemental analysis showed 0.56 wt.-% nitrogen content. This can lead to undesirable ammonia formation in the steam cracking process.

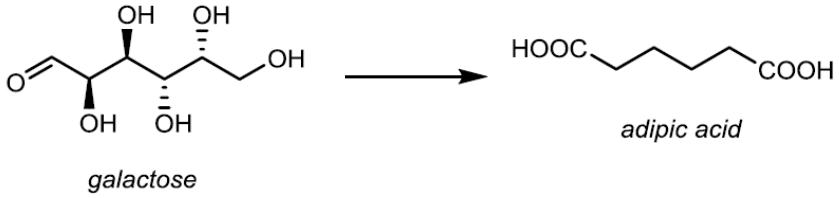
4.2 A one-step-pathway of directly steam-cracking the botryococcenes as process concept was developed and based on process simulations using the software Aspen PlusTM a steam-cracking bench-scale-plant was designed. The actual feedstock was planned to be botryococcenes blended in hexadecane. Operations were therefore carried out with hexadecane as model feedstock. Using hexadecane as the feedstock, ethylene and propylene yields as high as 20% and 11%, respectively, were obtained. The results show that the bench-scale plant, implementing the one-step pathway of steam-cracking, is suitable for cracking larger hydrocarbons into a gas phase rich in ethylene and propylene.

The unexpectedly high nitrogen content of 0.56 wt%, however, makes an industrial route from EHC to ethylene and propylene as platform chemicals unrealistic. It is favourable to focus on extracting the valuable chemicals of *Botryococcus* hydrocarbons, purifying them and utilising them without or with only small modifications in the molecule structure.

4.3 The polysaccharide fractions obtained in WP3 were hydrolysed (with acid) to obtain the monosaccharides. The main EPS sugars were galactose and fucose. Other sugars, i.e. arabinose, rhamnose, glucose, mannose, galacturonic acid and glucuronic acid, were only present in small amounts

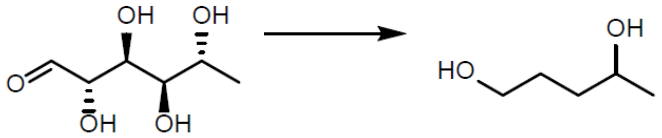
4.4 The original aim of this deliverable was to separate the monomeric sugar mixture into the individual monosaccharides. Due to, among others, a lack of technologies to perform sugar separation separation at scale the focus was shifted to analysis of the properties of theEPS from *B. braunii*. The results obtained demonstrate that the EPS is a long chain polymer and due to its pseudo-plastic behaviour it has potential to be applied in e.g. wall paints.

4.5 Galactose was converted into adipic acid (below) in a multistep synthesis. Different synthesis routs were tested. The overall yields were, however, always significantly below 50%.



galactose *adipic acid*

4.6 1,4-pentanediol (Scheme 2) was successfully synthesised from rhamnose in multiple steps, although the overall yield was low and must be significantly improved.



rhamnose *1,4-pentanediol*

4.7 The conversion of C6 sugars into furans is well known. In recent years, Avantium has developed the YXY-process (Figure 2), which starts from sugars to produce 2,5-furandicarboxylic acid (FDCA), which can be polymerised to polyethylene furanoate (PEF).
In October of 2016 Avantium and BASF formed a joint venture to commercialise the production of PEF.

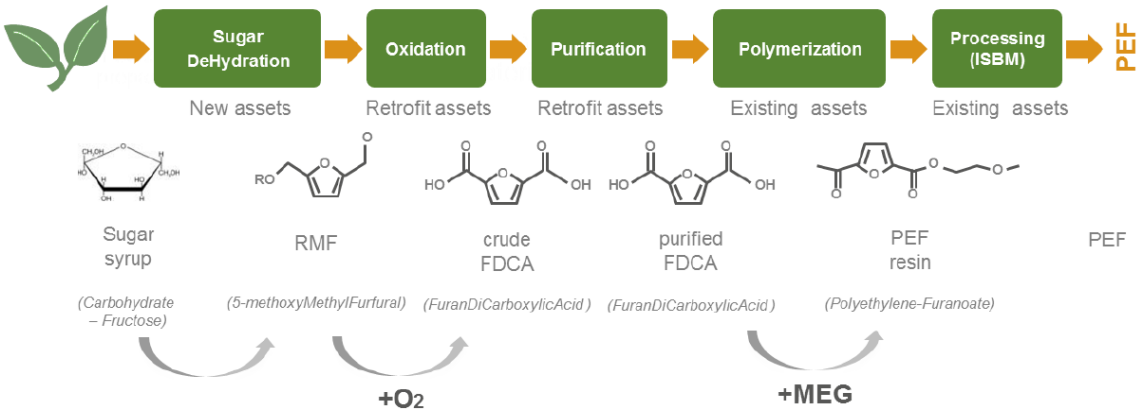
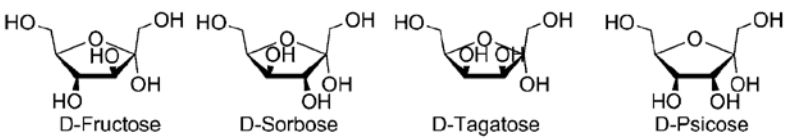


Figure 2: The YXY process

For effective dehydration to furans, it is favourable to use ketohexoses as feedstock (scheme below). It was discovered that psicose was the most favourable feedstock for the production for alkoxymethylfurans.
Since it is clear that ketoses are the favoured feedstock, any algae aldoses (glucose, galactose) should be isomerised to ketoses. The isomerisation of glucose to fructose was researched in detail, providing the limitations of this reaction. The sugars in these equilibrium mixtures should be separated and purified. Extensive research was thus performed on the separation of fructose (a ketose) from glucose (an aldose). The selective dissolution of fructose from solid sugar mixtures was patented.
Due to the small amount of available *B. braunii* carbohydrates in the earlier stages of the project, no experiments have been performed with sugars obtained directly from algae.



4.8 The polyester of 1,4-pentanediol and adipic acid was prepared for the first time and resulted in a sticky polymer. In addition, other biobased building blocks were tested in combination with adipic acid and 1,4-PDO, resulting in different material properties.

4.9 The unavailability of sufficient amounts of purified algae based glucose prevented the synthesis of PEF directly from algae based sugars. This meant that we were not able to look into the effects of using these sugars on the quality of the produced PEF. For processing available sugars from other sources were used.

4.10 PEF was successfully used for fibre production (Figure 3), with good spinability, potential for stretching and a resulting fibre with good mechanical properties and acceptable abrasion resistance. The results show PEF as a good candidate for industrial textile applications.

Figure 3: Fibres prepared by Lankhorst Euronete from PEF provide by Avantium

WP5 Process demonstration at pilot scale

Overall objectives of WP5
Demonstrate the capability of the optimised process (cultivation, integrated extraction and product separation of exopolysaccharides and hydrocarbons), at pilot scale under industrial representative conditions, based on the technology and methodologies developed in the previous WPs

Key findings and conclusions

5.1 It is possible to cultivate the *B. braunii* sugar strain in a continuous regime and also to accomplish a milking process. Nevertheless, the efficiency of the process has to be remarkably improved. Thus, further improvements are necessary in order to achieve a stable and continuous process. With respect to the commercial scale production of these algae sugars, the cost of production, harvesting and purifying algae sugars at this point still looks far away from being competitive. Further research is still required to achieve close to price parity with bulk polymers which typically have a cost price of ≤2500 $/ton.
Upon SoniqueFlo treatment, the B. braunii Showa broke apart and lipid was released. The potential for milking the matrix for hydrocarbon (so leaving cells alive) would not be a viable route for efficient extraction at high volume due to cell death caused by the treatment.

5.2 Within the project it was not possible to provide sufficient amounts of purified algae monosaccharides as feedstock for pilot scale polymer production. The choice was made to produce polyethylene furanoate (PEF) from commercial sugars in order to provide material to Lankhorst Euronete for materials testing. For adipic acid pilot scale production of fibres could not be performed. Apart from the lack of biobased feedstock, also the yield of the conversion of galactose into adipic acid was too low to perform this work in the available pilot equipment.

5.3 The *Botryococcus* hydrocarbons can technically be cracked into smaller fractions, namely ethylene and propylene which can be used as feedstock for polyethylene and polypropylene. Ethylene and propylene are, however, relatively inexpensive commodities (<€1000/T). As it stands, though the production of *B. braunii*, EHC appears too expensive for such a low value application. Furthermore, the N content in the oils needs to be reduces. The EHC, however, appear to be much more valuable as squalene/squalane type

application.
The EPS can be hydrolysed to monosaccharides (galactose, glucose, fucose), which require separationand purification before use in various chemical applications regarding monomer production. The production of adipic acid, which is applied in the production of Nylon 6,6, still requires a large increase in yield before it could be considered for scale up, since it would have to compete with existing fossil based adipic acid. At the moment, the production of purified algae based sugar feedstock for this process seems far too expensive to be considered economically feasible. Both producing the EPS and separating/purifying the different sugars still require significant research efforts.
2,5-Furandicarboxylic acid (FDCA) is a very promising novel monomer for especially polyester synthesis. It is viewed as a high-potential replacement for fossil based terephtalic acid (PTA), which is used in bulk polymers, mainly PET. When PTA is replaced by FDCA, a polymer comparable to PET is produced, namely PEF. Avantium and BASF have started a Joint Venture named Synvina to commercialise the production of PEF from sugars. This shows it has great promise, but it remains important that the feedstock price is acceptable. The same issues regarding feedstock for adipic acid apply to FDCA.
1,4-Pentanediol (1,4-PD), which can be produced from certain deoxygenated sugars (i.e. fucose), is a completely novel chemical. Some promising polymers have been synthesised, meaning this monomer has potential, but it is still too early to look into upscaling production of 1,4-PD. Apart from this, the same issues regarding feedstock production and purification as for FDCA and adipic acid apply here.

In general it can be said that much more research is required to lower the price of the algae based sugars before commercial bulk applications can be performed.

WP 6 Process integration, sustainability assessment and market analysis

Overall objectives of WP6

Integrate the different steps in the process, optimise the process chain and assess the economic, environmental and social impacts of the entire process.

Key findings and conclusions

6.1 Microalgae-derived biobased products (i.e., biofuels, biochemicals and biopolymers) have received increasing attention as one of the solutions to the continued and growing dependence on fossil resources. In this context, *Botryococcus braunii* offers intersting opportunities as it secretes unique extracellular polymers. Amathematical model for the microalgae-based production of extracellular polysaccharides (EPS) and hydrocarbons (EHC) and their conversion to respective biopolymers was developed. This model enables the evaluation of the economic feasibility and sustainability.

6.2 Eight scenarios were selected, simulated and evaluated with the developed model.
The 8 scenarios are:

	Product	Proposed volume	Optimised sales price	Market price range	GO/ NO GO
S1a	EPS	20.000 T	**€5/kg**	€7-15/kg	
S1b	EPS	200 T	**€43/kg**	€15-20/kg	
S2a	EHC	10.000 T	**€6/kg**	€4-6/kg	
S2b	EHC	500 T	**€20/kg**	€30-45/kg	
S3a	1,4-PDO	2.000 T	**€17/kg**	€2-5/kg	
S3b	2,5-FDCA	10.000 T	**€11/kg**	€1/kg	
S3c	Adipic acid	10.000 T	**€9/kg**	€1,5-2,5/kg	
S4	Ethylene	100.000 T	**€4/kg**	€0,5-1,1/kg	
S4	Propylene	100.000 T	**€4/kg**	€0,75-1,3/kg	

EPS = ExoPolySaccharide; EHC = ExoHydroCarbon; PDO = Pentane DiOl; FDCA = FuranDiCarboxylic Acid

For each scenario, the respective flow sheets were modelled in 3 operating areas:

· Area A: Preculture preparation and cultivation/production of microalgal biomass

· Area B: Recovery/milking, separation and drying of the EPS and EHC.

· Area C: Conversion of EPS and EHC to building blocks and biopolymers as end-products

6.3 From the analysis of the scenarios it was found that the major hotspot for both capital and operation expenditures is the cultivation of microalgae in the photo-bioreactors (Area A), followed by the milking/separation step (Area B). From the selected key-process parameters, the biomass specific growth rate and the EPS/EHC yield are the ones with the major effect on the production cost, followed by the milking and drying/separation efficiency.

6.4 A database with market value for the different *B. braunii* products has been completed and used –in addition to technical feasibility assessed in WP 3&4- to select the 8 scenarios reported above. In addition to the economic and environmental analysis performed by CERTH and OWS, the value proposition for each scenario was also completed: what are the unique sales arguments for each scenario? Additionally, an 'Optimised sales price' (= sales price leading to a payback of max 5 yrs and IRR> 20%) for each scenario was computed

Scenarios that consider EPS and in particular EHC as the final products are the most promising ones, because of more simple plants and higher values of the end products. The remaining four scenarios (S3a, S3b, S3c and S4), where the EPS and EHC are converted to (building blocks for) biopolymers, seem not viable under the current conditions. This is due to the more complex processes (additional equipment needed) and the low value end products.

With respect to the commercial scale production of algae sugars (monosaccharides) the cost of production, harvesting and purifying algae sugars at this point still looks far away from being competitive. Further research is still required to achieve close to price parity with bulk polymers which typically have a cost price of ≤2500 $/ton

The in SPLASH developed cultivation and milking technologies have the potential to operate economically, in particular for EHC, provided that all the involved processes will be optimized and the targeted products are of higher than commodities value

6.5 The environmental impacts were determined by life cycle assessment (LCA). The impacts of dry EPS and dry EHC are dominated by the energy use of isolating and, especially, drying the EPS and EHC. The

SPLASH adipic acid and, to a lesser extent, FDCA production pathways can be considered environmentally competitive, especially when using renewable energy for all power and heat needs of the SPLASH developed production steps. Without additional process improvements, SPLASH 1,4-pentanediol, ethylene, and propylene are unable to compete against their mass-produced competition on the level of 'environmental impact'.

6.6 When developing new products, it is important to have clear quality control objectives, translated into specifications. As most products considered in SPLASH are new products, setting specifications has been inspired by proxy commercial products (= products with similar functionality).

Most specs are dealing with high purity (end products contains almost exclusively the targeted molecule), low water content and almost absence of heavy metals. Specifically for cosmetics applications, additional specs on microbiology and organic certification will be needed. Building blocks for (bio)polymers needs an extremely high purity (>99%) and absence of other (organic) contaminants that may interfere with the polymerisation process. Finally, there are also some specific specs for biobased products, mainly related to certifying the biobased content.

6.7 To estimating the impact of SPLASH on the society: biobased economy, employment and environment first, a 'big data' simulation was made:" imagine a total substitution of petroleum by biomass and in addition a sufficient food production for 11 billion people". What would that mean on needed quantities, on (arable) land requirements? A benchmark with wheat and sugarcane indicates the huge –if not impossible- challenge when considering traditional crops. This indicates already the potential importance of microalgae in a transition scenario towards a biobased economy or a 'microalgae-based economy'. *Botryococcus braunii*, the algae studied in SPLASH, has a specific role, as it excretes unique polymers leading to biobased chemicals. The EHC produced by *B. braunii* can even be labelled as 'biocrude'.

Such a total substitution of all crude oil and a sufficient (caloric) food production for 11 billion people will impact employment, as it requires >10 million FTE for production alone. Compared to the 4-6 million production labour in the oil industry, it creates an additional employment potential.

Regarding the impact on the environment from different perspectives (water, carbon, nutrients and energy requirements, land use, eutrophication, GMO, algal toxicity) advocates for a closed production system for microalgae, as it controls the in- and outflow better then open ponds.

On the 'big data' scale however, the huge impact on energy requirement and nutrient need for a total substitution of crude oil and a sufficient food production in a clear point of concern.

Energy requirements for algae production should be significantly reduced and for becoming a major crop.

WP7 Dissemination, exploitation and intellectual property management

Overall objectives of WP

Dissemination, exploitation and technology transfer of project results to the relevant stakeholders including policy makers, industry and society including the management of intellectual property rights (IPR).

Key findings and conclusions

7.1 A communication and dissemination plan has been set up in coordination with the project coordinators (NOVA, DLO). The plan describes amongst others the appearance of the internal and external website, the leaflet of the project as well as a concept of the International Conference "European Roadmap for an Algae-Based Industry" in 2016. It also gives an overview of publications and visited events.

7.2 During the project a full update of the scientific state of the art and IP positions has been generated in collaboration with all partners. A mechanism to safeguard against accidental disclosure of valuable IP has been implemented. During the SPLASH two patents have been published by Furanix Technologies BV, a daughter company of Avantium Holding BV: The exploitable results of SPLASH for Avantium are the exploitation of the patent WO 2015/133902 A1 "A process for the catalyzed conversion of Psicose into 5HMF or an alkylether thereof" and the second patent "Process for the preparation of a fructose-rich solution from a solid composition comprising fructose and glucose

7.3 A cloud storage database was developed as well as a website (www.eu-splash.eu). After the end of the

project the project website will be available for 5 five years for all partners of SPLASH as well as for the public. In the period January 2013 till 3 February 2017 the total amount of page views is about 27,300. This is comparable with websites of e.g. the EU-project MIRACLES. Most of the visitors came from Germany, The Netherlands and USA. The most visited pages were the start page (12,600 clicks) of SPLASH and the page of the consortium (3,900).

7.3 Three press releases have been published. A first press release was written and submitted in December 2012 to more than 1.700 press contacts which caused a high rate of page visits. The objective was to inform about the project start as well as the project partners. Since publication of the press release it has been downloaded more than 2,800 times. The 2nd press release has been published in March 2015 including results of the stakeholder survey analysis. The topic was "SPLASH Stakeholder Analysis reveals opportunities for collaboration in third generation algae bioplastics". It has been written together with PNO and sent up to 2,000 press contacts. The press release has been downloaded for 1,300 times at the SPLASH website. A 3rd press release has been published in April 2016. The press release "EU ALGAE stakeholders release ground-breaking agenda to develop the industry" has been written together with PNO and has also been sent to 2,000 press contacts. It has been downloaded for 470 times.

7.5 A leaflet in English was prepared. It is also available as a PDF download at the website of SPLASH and has been downloaded 700 times.

7.6 The project brochure is available in different languages (English, French, German, Dutch, Spanish) and is available for download. The top four downloads numbers are as follows: SPLASH brochure – English language: 579 clicks, German edition: 371 clicks, French edition: 324 clicks, Spanish edition: 288 clicks.

7.7 An international conference on the use of microalgae in the industrial biotechnology to promote the results of this project as well as to get in contact with other projects and stakeholders in this field and to find partners for the exploitation of the technical innovations was co-organized and SPLASH partners provided scientific input.

- International Conference "European Roadmap for an Algae-Based Industry" in Olhão, Portugal on 6 - 8 April 2016 with more than 100 participants. SPLASH partners presented the latest results of the SPLASH project via oral presentations as well as by posters.
- a white paper on industrial algae production for the European Economy State of the Art of algae production in Europe was prepared by the organisation

7.8 The proposed business and exploitation plans have been designed to integrate maximally the cost and economical value and a 'feasibility' dimension, derived from the results of the stakeholder analysis. As SPLASH is a rather early stage project, the Exploitation Plan has allowed for the identification of new Consortia to propose follow-up projects as selected by the Business Plan.

- More than 80% of the results will be further valorised by the result owners.
- There is a strong support amongst the SPLASH partners for a follow-up project.
- There is a strong opportunity for such a follow-up project.
- The follow-up project application will be entirely product-driven

7.9 The SPLASH project started at a Technology Readiness Level of 2-3 and brought this to a TRL of 4 and in some cases 5. This means that most of the steps involving the understanding of the *B. braunii* genome, the growth and extraction of B. braunii products and the downstream processing of those are now validated at lab scale or even in relevant environment. This also means that the SPLASH results are not yet ready for the market. The Consortium decided during the final General Assembly to edit a new project proposal designed around the production and valorisation of *B. braunii* exohydrocarbon, as this product is unique to B. braunii and has economical potential.

7.10 A large group of stakeholders has been identified and stakeholder analysis has been performed
Approximately 650 organisations were approached and approximately 10% participated in the survey.
The stakeholder analysis has provided a first insight in the extent to which the algae based bioplastics platform as developed by SPLASH is in line with stakeholder requirements. In particular it provided information on

stakeholders' attitude, interest and the criteria they use to assess the added value of SPLASH. It also proposed a general stakeholder management strategy based on stakeholders' respective positions.

7.11 SPLASH organized a very successful Summerschool, adjacent to the "Microalgae Process Design: from cells to photobioreactors" in cooperation with Wageningen graduate school VLAG, Wageningen University: Bioprocess Engineering, Wageningen Research: Food & Biobased Research, AlgaePARC from 18 – 20 July 2016. The course was aimed at PhD candidates, postgraduate and postdoctoral researchers, as well as professionals, that would like to acquire a thorough understanding of microalgal biorefinery. The summer school attracted 25 participants from all over the world.

Address of the project public website, as well as relevant contact details.

Project public website
www.eu-splash.eu

Contact details
Project coordinator
Dr. Lolke Sijtsma
Wageningen Research, Food & Biobased Research
P.O. Box 17, 6700 AA Wageningen
Tel +31 317 480220
e-mail: lolke.sijtsma@wur.nl