1 General Introduction

1.1 The global phosphate cycle

Phosphorus (P) is a key element for life. As phosphate (abbreviated as P_i or PO_4^{3-}) it is part of ATP, phospholipids, DNA, RNA, and bones [1]–[3]. In the environment, P_i occurs as phosphate ore which is mainly available as various types of apatite [$Ca_5(PO_4)_3$(F, Cl, OH)] such as fluorapatite [$Ca_5(PO_4)_3F$] and hydroxyapatite [$Ca_5(PO_4)_3OH$] [4]–[6]. Further phosphate rocks may also contain a wide range of contaminations, such as cadmium, uranium, radium, lead, and arsenic [4], [6] depending on the geographical location of the reserve. The largest P_i ore reserves have so far been found in Morocco, China, and Norway [7], [8]. Naturally, P_i from the ore slowly erodes into water streams and soil. Plants can absorb P_i, partially followed by an uptake of the plant by animals. Through animal excretion or the die-off and decomposition of animals and plants, P_i returns to the soil. This part of the P_i cycle is retreaded several times before it washes out of the local environment. P_i-containing material then flows via rivers and lakes into the sea. Meanwhile, this material is also repeatedly recycled between aquatic organisms and sediments. Sediment oceanic P_i can form phosphate ore again. Nevertheless, the whole process takes ten to hundred million years [1], [9]. Therefore, phosphorus belongs to the elements with the slowest biogeochemical cycles among all elements [6].

Humans interfere with this natural cycle by mining phosphate on a large scale removing four times more phosphorus compared to the natural erosion [5]. More than 80 % of this mined phosphate is used in fertilizer [10], [11]. It enhances the crop yield and is thereby essential for the food supply of a growing world population [11], [12]. Plants take up parts of the provided P_i and use it for growth. Subsequently, this P_i returns via animal and human nutrition and other intermediate steps (see also chapter 6.1) back to the soil and thereby back to the natural cycle [1], [9]. Both cycles (natural and human-derived) described here are shown in Figure 1.

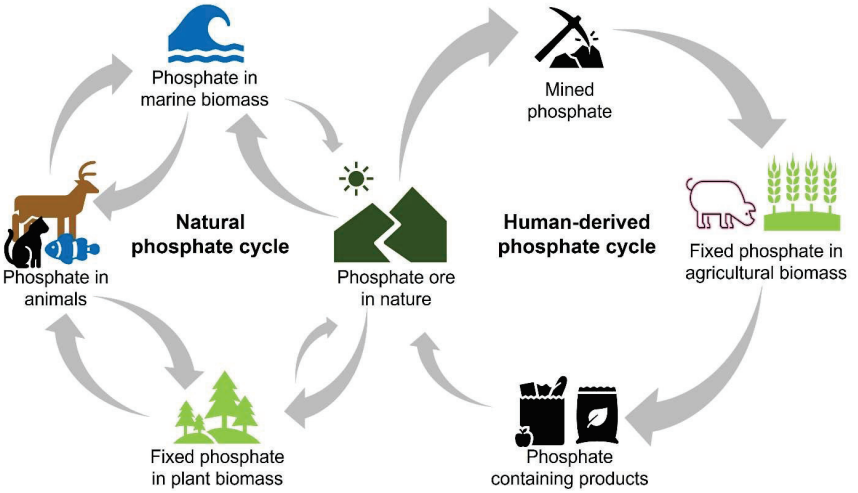

Figure 1 Natural and human-derived phosphate cycle. In the natural P_i cycle, P_i from the ore erodes into water streams and soil. Plants and algae can absorb P_i followed by an uptake of the fixed P_i by animals (terrestrial and marine). Through excretion or die-off, P_i returns to the water and soil, and after millions of years, P_i ore is formed again. Humans interfere with this natural cycle by mining phosphate on a large scale. Often this P_i is used as fertilizers and feed additives for enhanced crop and animal product yield. Via the usage of various P_i-containing products, P_i returns to the soil and thus to the natural cycle [1], [9]. The size of the arrows indicates the strength of the P_i flow (small arrows = slow flow, which takes a long time; large arrows = strong and fast flow). The information in this figure is based on Ref. [1], [9]. The figure including the pictograms was created by using PowerPoint.

1.2 Current phosphate situation and recycling strategies

Due to the increased utilization of phosphate by humans, the natural phosphate cycle is broken. In 2014, the EU listed phosphates as critical raw materials [13], adding elemental phosphorus in 2017 and all kinds of phosphorites in 2020. This is a fast development. The National Mineral Information Center of the U.S. numbered global phosphate rock mining volume at 220 million tons in 2022. The existing reserves were estimated at 72 billion tons at the same time [14]. Based on that data the global deposits would be exhausted in approximately 330 years. However, it must also be considered that the phosphate demand is increasing to meet the food requirements of a growing world population. Therefore, the time until all minable P_i rock reserves will be depleted is shrinking. Experts estimated that the available resources will last for the next 200-500 years

[15], [16]. Phosphate reserves differ in their quality. They are classified as high-grade ore (26-35 % as phosphorus pentoxide (P_2O_5)), medium-grade ore (17-25 % P_2O_5), and low-grade ore (12-16 % P_2O_5) [5]. The initial beneficiation of this ore increases the purity by for example removing sand and clay. Typically the mined phosphate rocks contain 30-32 % P_2O_5 [6]. Two types of processes are predominantly used for further processing of the P_i rock: The wet acid digestion process and the direct thermal conversion [4]. The prior process is used to convert 85 % of the P_i rock into high-grade phosphoric acid [4] by crushing the dried P_i rocks and mixing them with sulfuric acid (H_2SO_4). Here, calcium from the P_i rock reacts with the H_2SO_4, forming calcium sulfate ($CaSO_4$), commonly known as gypsum, and phosphoric acid (H_3PO_4) [17]. The crude phosphoric acid is purified by solvent extraction [18]–[20]. The direct thermal conversion is applied by producing high-grade phosphoric acid directly from P_i rocks. Here elemental phosphorus is condensed and burned into P_2O_5 at temperatures of 1650 to 2760 °C. Afterwards, P_2O_5 is hydrated to phosphoric acid. Thereby, phosphoric acid with concentrations of 75-85 % (v/v) is produced. This is required for high-grade chemicals and other non-fertilizer products [17].

The importance of phosphorous is also reflected in the planetary boundaries where it has been listed since it was first proposed in 2009 by Johan Rockström and a group of 28 internationally renowned scientists [21], [22]. Planetary boundaries are defined as ecological global limits that, if exceeded, endanger the stability of the global ecosystem and thus life. These limits include topics like climate change, freshwater supply, land system change, and biochemical flows of phosphorus and nitrogen. In 2015, the boundary of phosphorus was crossed so the phosphorus situation is now classified as highly risky. This means that humanity has left its safe operating space regarding P_i and if the situation does not improve there will be a high risk of serious consequences for life itself [23]. Additionally, the accelerated release of phosphate leads to several problems. P_i rocks contain toxic or radioactive elements like arsenic, cadmium, and uranium [24]. For low-grade applications, such as the commonly used Triple Superphosphate or Superphosphate fertilizers, 50 to 360 mg uranium per kg fertilizer was measured [24], [25]. This results in an accumulation of radioactive and toxic elements in the farmland and the plants, affecting food safety, plant growth, soil microflora, and water quality [26], [27]. Furthermore, CO_2 emissions, energy, and water costs during mining and transportation must be considered as they contribute to pollution. P_i mining is therefore not an environmentally friendly process and in times of global warming and rising energy costs, it is no longer in keeping with the times. Moreover, it deconstructs the environment and natural habitats of animals and plants are destroyed. Since the habitat of many animal and plant species is already limited, this can increase the loss of biodiversity further. In addition, by phosphate mining, P_i-rich waste leaks into lakes leading to eutrophication [28]. With

eutrophication, the increased enrichment of nutrients leads to rapid bacterial and algae growth competing against water plants and animals (*e.g.* fish) for oxygen in the water [29]. As a result, many animal and plant species in the affected water body die off, leading to a loss of biodiversity also in marine habitats. This human-derived, global problem is well known even though it is largely unresolved [30]. Moreover, there is a substantial P_i-loss while using P_i-rich fertilizer as plants can only take up a fraction of the P_i-rich fertilizer introduced. The rest of this fertilizer can be washed out which further intensifies eutrophication.

Currently, 75 % of the discovered global P_i reserves are in Morocco and China [14]. Most of the phosphorite imports into the EU originate from Morocco, Russia, and Finland [31]. This fact also adds a political component to the topic especially since relations with Russia have deteriorated as a result of the Russian-Ukrainian war. However, in 2021 the Norge Mining Company issued a press release about a P_i reserve discovery in Storeknuten/Bjerkreim - Norway. They estimated the deposit at 910 million tons of the critical raw materials phosphate, vanadium, and titanium. By the exclusion of two lower-grade areas of phosphate, 410 million tons containing high-grade 3.36 % (w/w) P_2O_5 were found [8]. The European Raw Materials Alliance (ERMA) is supporting Norge Mining in the exploration and production of the minerals and their derivatives in Norway to increase the EU's autonomy on critical raw materials including phosphate [32]. Nothing is yet known about the start of the P_i mining there. Nevertheless, the global phosphate reserves are decreasing, and mined ones accumulate after usage in ponds, lakes, and the ocean where it has harmful effects on the environment. New strategies have to be developed to recycle and reuse the already available phosphate. Due to that, in 2017 Germany enacted the Sewage Sludge Regulation in which the utilization of sewage sludge, sewage sludge mixtures, and sewage sludge compost especially as fertilizer is regulated. In this regulation, the recycling of sewage sludge containing phosphorus from 2029 onwards is specified [33]. By then, 50 % of the amount of phosphate in sludge and 80 % of phosphate from sewage ash have to be recovered [34]. The public acceptance of using phosphate-rich sludge directly is low due to potential environmental and health risks based on contained heavy metals, pollutants, and pathogens. Therefore, recycling methods have been developed in the last years acting on different points of the processing, usage, and waste chain [35]. For example in a wastewater treatment plant (WWTP), bacterial sludge already removes about 20-50 % of the phosphate contained in wastewater in a traditional setup through anabolic uptake [6], [35]. However, this often does not remove enough phosphate, as biological methods are less efficient than chemical processes [6]. Furthermore, the P_i is then not freely accessible but must be processed from the P_i-containing sludge. At the moment the preferred strategy is the combustion of sewage sludge followed by the recovery of the ash containing P_i [34].

Although there are several prominent technologies for phosphorus recycling and recovery from sewage sludge and ash, only a few have had a small breakthrough up to now (see Ref. [35] for a complete overview of all technologies) and were transferred to larger pilot plants. The majority of the implemented processes with names such as ANPHOS, NuReSys, and the Stuttgarter process recover P from sewage sludge as struvite [35]. Struvite (NH_4MgPO_4 x 6 H_2O) is a mineral that is hardly water soluble, has a low metal content, and contains phosphate, nitrogen, and magnesium. These characteristics make struvite a suitable slow-release fertilizer, although it is not as suitable as conventional fertilizer [6], [35]. All these processes use the direct method in which P_i dissolved in the water phase is precipitated as struvite. However, this method is not applicable to P_i bonded in solid particles [36]. Struvite precipitation is highly effective for waste streams with little solid particles, and high phosphate content (> 50 mg/L) [6]. Thus, mentioned such technologies cannot be used for all WWTPs [35]. However, it is currently the leading technology to recover P_i and ammonium at the same time enabling its reuse as fertilizer [37].

An alternative is the mono-combustion of the sewage sludge followed by treatment of the received ash. This strategy has the advantage of destroying possible organic pathogens and toxins within the sludge. However, mono-combustion results mainly in apatite [$Ca_5(PO_4)_3(F/Cl/OH)$] and whitlockites [$Ca_9(Fe/Mg)(PO_4)_6PO_3OH$]. These are phosphate minerals with low availability for plants thus these compounds are not applicable as fertilizer [36]. The process called TetraPhos developed by Ramondis Aqua makes sewage ashes recyclable [35], [38]. To this end, sewage sludge ash is dissolved in phosphoric acid, and sulphuric acid is repeatedly used as a fresh source of acid to leach the P_i out of the ash further. By several steps of separation and treatments, pure phosphoric acid and gypsum as a byproduct are produced. Thereby, more than 80 % of the ash contained phosphorus is recovered. It is especially suited to leach phosphate from iron-rich ashes. The produced phosphoric acid has a very low contamination level, making it suitable for industrial applications. Besides gypsum as a side product, the process also allows the recycling of iron and aluminum salts [38], [39].

Struvite and phosphoric acid are so far the two main products generated in various processes by recycling phosphate or phosphorus from sewage sludge and ash. However, all these processes have their advantages and disadvantages such as byproducts or production costs. It is therefore important to continue research into alternative recycling strategies.

1.3 Polyphosphate and its application

Until now, mostly technical processes have often been used for P_i recycling. However, in wastewater treatment plants microorganisms are increasingly being used besides other strategies

which can selectively absorb the P_i and often store it intracellularly as polyphosphate (polyP). polyP is the polymer of P_i linked by phosphoanhydride bonds and can be found in all living organisms from bacteria to humans. As a polyanion, it can chelate cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+} [40], [41], or it can form complexes with other molecules like proteins or nucleic acids [40]. Besides its complexation ability, polyP is supposed to be an energy storage *in vivo* and helps for example with the detoxification of heavy metal cations [40]. In bacteria, polyP is part of cell mobility, biofilm formation, and virulence. Furthermore, it takes part in the blood coagulation cascade in animals and humans [40]. It may exist as a linear, cyclic (also known as metaphosphate), or branched (ultraphosphate) structure (Figure 2) [42], [43]. The main structure of polyP in cells is linear. However, the origin of the other two structures has not yet been definitively clarified. Neither the biological function nor synthesizing or degrading enzymes of these polyPs are known. Recently, it was investigated if cyclic and branched polyP can occur naturally in biological systems or if their detection is rather analytical artifacts [43], [44].

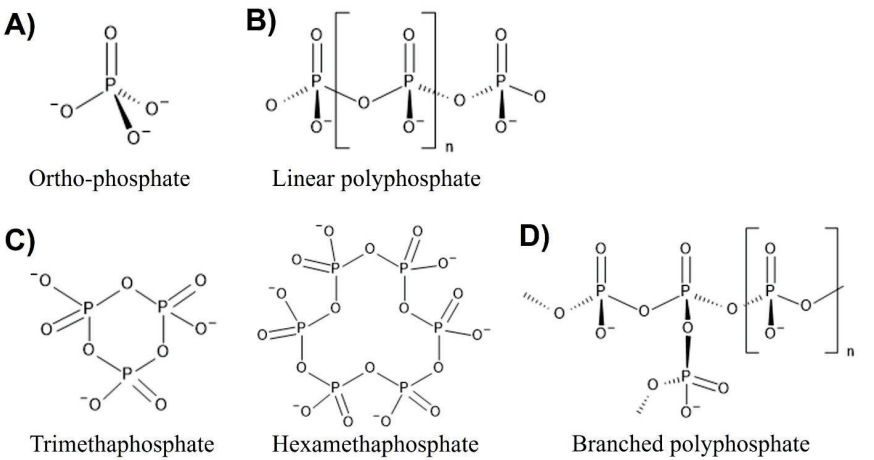

Figure 2 Molecular structure of P_i and polyP. A) single phosphate molecule, B) linear structure of polyP, C) most common structure of cyclic polyP (also known as metaphosphate), D) branched polyP (ultraphosphate). n can range from 0 up to several hundred. (D) can have one or more branching points that can be at different phosphate groups of the chain. The information in this figure is based on Ref. [43], [45]. The structures were generated with Biovia by Dassault Systems.

Cyclic polyP is stable in water [44]. ^{31}P solid-state NMR data of *Xanthobacter autotrophicus* show that small cyclic polyPs can bind to enzymes like ribonuclease A and nicotinamide adenine dinucleotide (NAD) kinase [44]. However, the occurrence of cyclic polyP in biological systems has to be critically evaluated because linear polyP has the ability to form rings spontaneously [46], which means that it could also have formed during sample preparation in the aqueous system. Most common cyclic polyP molecules consist of three or six P_i molecules and are probably limited to eight P_i molecules [34], [44]. However, the biological role of cyclic polyP is still unclear. In chemistry, they can be synthesized by heating phosphoric acid [47] and especially sodium hexametaphosphate is well known as Calgon for water softening. Moreover, it is used as a corrosion inhibitor, and in cosmetics, and personal-care products.

Since 1950 it has been reported that branched polyP (ultraphosphate) refers to the antibranched rule. This rule signifies that the branching points of the ultraphosphates become unstable and hydrolyze spontaneously at a high rate as soon as polyP dissolves in water [43], [48]. Although this rule lacks experimental backup, this opinion has persisted until shortly. Dürr-Mayer *et al.* put this rule into question in 2021 by synthesizing branched polyP and measuring hydrolysis half-lives up to days. Further, they discovered a rearrangement by an enzyme that linearizes the branched structures [43].

However, the linear polyP is the most common and investigated structure and can be found in all living organisms [49]. It has two up to several thousand P-subunits [50]. Further linear polyP can differ in its counterion composition. This together with the chain length of the polymer defines the molecular weight of polyP. PolyP has several unique physicochemical properties which are also influenced by the counterions and the chain length. These properties define a broad spectrum of applications [40]. Nonetheless, 80 % of the mined P_i and the resulting polyP continue to be applied as one of the most important compounds of fertilizer [10], [11], [51], [52].

As polyP beyond this is, *e.g.,* hydrophilic and non-flammable, it is also often used in flame retardants [40]. Moreover, it is applied as a food additive *e.g.* as a preservative and stabilizer because of its biodegradable, bacteriostatic, and mild proton donator characteristics. Additionally, it acts as a cation exchanger and a pH buffering agent [40], [47]. As only the two outer phosphates of polyP buffer the pH, more often polyPs with short chain lengths are used for that purpose. PolyPs with a chain length of two and three P-subunits (polyP2 and polyP3) are widely applied to increase the water binding capacity in meat. It is suggested that PolyP2 separates myosin from actin. As a result, myofibrils are swelling which leads to increased hydration for example in muscles [53], [54] improving tenderness of processed meat. Both short cyclic polyPs like

trimethaphosphate or hexamethaphosphate, and ortho-phosphate, however, have less or no water-binding capacity [54]. Effects like pH buffering and water-binding are less prominent with increased polyP chain length, whereas the bacteriostatic effect and the ability to complex cations increase with longer chain lengths. This is why they are used more in the production of *e.g.* soft cheese.

1.4 Polyphosphate production

PolyP can be produced chemically and biotechnologically. In chemical synthesis, polyP is produced by heating monophosphoric acid (H_3PO_4). During a condensation reaction, intermolecular water is released and diphosphoric acid ($H_2P_2O_7$) is produced. Between 400 and 800 °C, polyP with different chain lengths is synthesized by further condensation reactions starting from diphosphoric acid [55]. H_3PO_4 is produced either within a thermal process or in a wet process. The wet process is common for the production of fertilizer and technical H_3PO_4. About 95 % of the total produced H_3PO_4 is generated by this process[56]. Compared to that, H_4PO_3 produced by the thermal process is purer and is used for food and special industrial applications but the process is more energy intensive [56].

In nature, a broad range of organisms such as algae [57], bacteria [58], [59], mammalian cells [60], [61], and yeast [49], [62], [63] are able to accumulate phosphate and produce polyP. This usually happens after a starvation period with little or no phosphate. Due to a subsequent excess of phosphate, they are taking up more phosphate than needed for living [47], [64]. The reason for that is unclear. Assumptions are ranging from phosphate [47]and energy storage [47] to stress protection [47], [65], [66]. Although different polyP-related enzymes play a role in various organisms, the general process is well preserved. They all developed strategies to deal with the decrease of phosphate and its final absence. However, in all these systems, a naturally or artificially induced starvation phase stimulates the increased and rapid uptake of phosphate from the environment. Depending on the organism different enzymes accumulate this phosphate as polyP within the cells. For example, Langen and Liss (1958) showed that after such a P_i starvation, polyP is produced naturally in *Saccharomyces cerevisiae* (*S. cerevisiae*) [41]. In 1962, they showed that the amount of produced polyP is not caused by reduced degradation of polyP but by enhanced polyP synthesis [63]. Recently, the so far highest polyP content of 28 % (per cell dry weight as KPO_3) in *S. cerevisiae* was reported [62].

1.5 Phosphate homeostasis in *Saccharomyces cerevisiae*

The polyP metabolism in *S. cerevisiae* is tightly connected to the P_i homeostasis including proteins for transport, gene regulation, re-mobilization, and P_i recycling. At least 25 yeast genes are directly involved in P_i homeostasis and are mostly regulated by the phosphate signal transduction (Pho-) pathway Figure 3 [57]–[59]. *S. cerevisiae* has four membrane-bound P_i-transporters that mediate P_i uptake and complement each other. Under P_i limitation the high-affinity H^+/P_i symporter Pho84p and high-affinity Na^+/ P_i symporter Pho89p are upregulated [70], [71]. Both transporters have a K_m value of 8-40 µM and are tightly regulated [67], [72] not only by the Pho-pathway and the connected intracellular phosphate amount, but also by other complexes, pathways, or the cell cycle [68]. Pho84p, the main high-affinity P_i-transporter under normal growth conditions, is most active at acidic pH whereas the second high-affinity transporter Pho89p mostly works under alkaline conditions [68], [72]. Pho84p is highly expressed at < 0.5 mM P_i, but becomes transferred to the vacuole for degradation when the external P_i concentration drops below 30-40 µM [73], [74].

In the absence of the high-affinity transporters, the low-affinity transporters, Pho87p and Pho90p with a K_m value of 200-800 µM and located in the plasma membrane ensure P_i uptake [68], [71]. Both are divalent anion Na^+ symporters and harbor a SPX (*S*YG1/*P*HO81/*X*PR1) domain at their N-terminus. These SPX domains control the flux of P_i via the transporter across the cell membrane in both directions by their interaction with *myo*-inositol pyrophosphates [71]. In contrast to the high-affinity transporters, the transcription of the low-affinity transporters is independent of the P_i level [71], [75]. However, under P_i starvation conditions these transporters are also endocytosed and are transported to the vacuole. Pho90p is the most important transporter under high P_i concentration conditions [75]. Further, Pho90p and Pho87p are involved in external P_i sensing [68], [75], [76]. At an intermediate P_i level, both high- and low-affinity transporters are expressed [77]. The cell membrane-bound Syg1p transporter with an SPX domain is predicted to be an exporter for P_i as this activity was shown for its mammalian homolog XPR1 with which the sequence of Syg1p shares 30 % identity [67], [78]–[80]. Nevertheless, the activity of SYG1 itself has not yet been demonstrated.

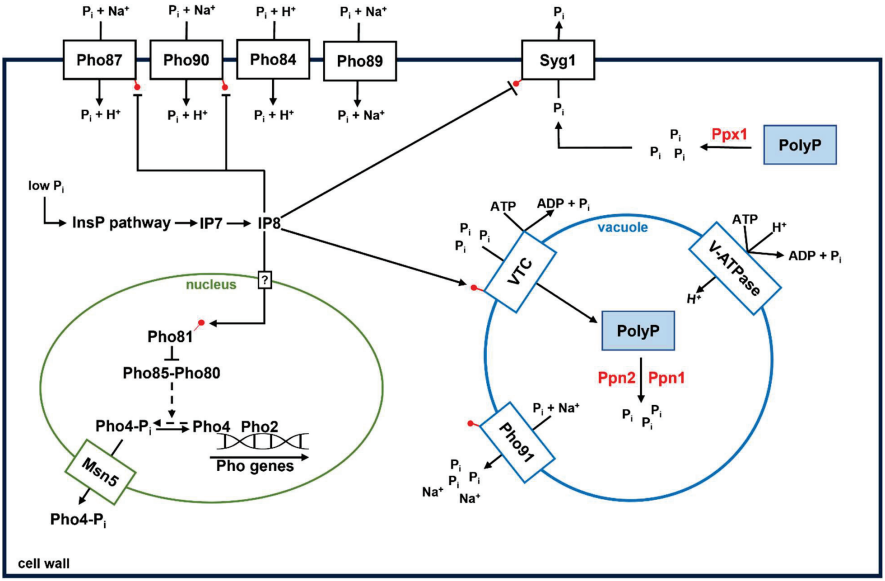

Figure 3 Scheme of the metabolic Pho-pathway with its central regulations in *S. cerevisiae*. The Pho-pathway regulates P_i homeostasis and polyP production in yeast. Red dots are SPX domains for the regulatory interaction with IP8. The signal transduction pathway is activated with low intracellular P_i concentration and takes place under aerobic and anaerobic conditions. Pho = genes of phosphate signal transduction pathway; P_i = free phosphate; polyP = polyphosphate; InsP = inositol phosphates; IP7 = diphosphoinositol (1,2,3,5,6)-pentakisphosphate; IP8 = bis-diphosphoinositol tetrakisphosphate; Msn5 = karyopherin; VTC = eukaryotic vacuolar transporter chaperon; Ppn1 and Ppn2 = polyphosphatases; Ppx1 = exopolyphosphatase. This figure is based on Ref. [45], [67].

Recent studies have shown that bis-diphosphoinositol tetrakisphosphate (IP8) activates (under P_i limiting conditions) the Pho-pathway shown in Figure 3 and not diphosphoinositol (1,2,3,5,6)-pentakisphosphate (IP7) as previously assumed [81], [82]. In the nucleus, IP8 binds to the cyclin-dependent kinase inhibitor Pho81p which prevents the complex formation of Pho85p-Pho80p [82]. Thereby, a phosphorylation of the transcriptional activator Pho4p is prevented. When phosphorylated, this activator is transported out of the nucleus by Msn5p. When dephosphorylated, Pho4p binds together with the transcriptional activator Pho2p to specific DNA sequences (CACGTG and/or CACGTT) found in the promoter region of the Pho-responsive genes triggering

their transcription [82]. Examples of such Pho-responsive enzymes are the exo-cellular acidic phosphatases Pho5p, Pho11p, Pho12p, and the high P_i-affinity transporters Pho84p and Pho89p. Further, the cyclin-dependent kinase inhibitor Pho81p and the polyP synthesizing complex are regulated via this pathway [81], [82]. In general, the transcription of all the genes regulated by the Pho-pathway is repressed when P_i is available and activated by P_i depletion [83].

1.5.1 Inositol and SPX domain

Phosphate homeostasis is strongly regulated in all organisms including bacteria, fungi, and mammalians [81]. However, it is not clarified in detail how organisms sense phosphate availability inside and outside of the cells yet [84], [85]. It is known that *myo*-inositol pyrophosphates (InsPs, IPs) have a key role in phosphate homeostasis [81], [84], [85]. This class of molecules contains up to six additional P-anhydrides attached to *myo*-inositol [85], [86] at different positions [84]. Together with CDP-diacylglycerol, InsPs are synthesized from *myo*-inositol via the phosphatidylinositol phosphate biosynthesis pathway in *S. cerevisiae*. The biosynthesis of InsP that is shown in Figure 4 takes place via an enzyme cascade starting from inositol-1,4,5-trisphosphate (IP3) which was previously produced together with diacylglycerol by hydrolyzing the lipid precursor phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2) by phospholipase C [86]. IP3 is then the substrate of Arg82p inositol polyphosphate multikinase forming inositol-tetrakisphosphate (IP4) and inositol-pentakisphosphate (IP5). Subsequently, Ipk1p phosphorylates IP5 to form inositol-hexakisphosphate (IP6) also known as phytic acid [86], [87]. This molecule is then the substrate for the enzymes Kcs1p and Vip1p to form IP7 and IP8 which have major regulatory roles in phosphate homeostasis and polyP production. The order of these two enzymes only determines whether 1-IP7 or 5-IP7 is the precursor of IP8. Both enzymes add one phosphate to the inositol ring. While Kcs1p can also synthesize PP-IP4 from IP5, Vip1p is a bidirectional enzyme that can not only convert IP7 to IP8 but can also reverse this reaction [86], [87]. Previous studies described the regulation of phosphate starvation by the intracellular IP7 level [87]–[89]. However, recent studies have shown that levels of IP7 and IP8 are strongly decreased under P_i starvation, and especially IP8 triggers the transcriptional P_i starvation response [81], [86].

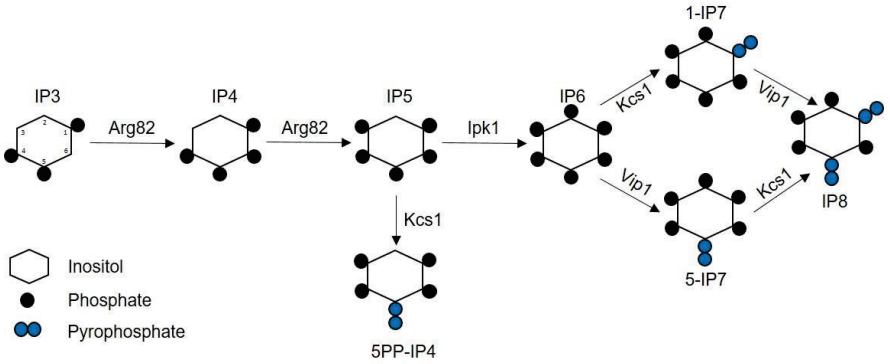

Figure 4 Inositol phosphate (IP) biosynthesis in *S. cerevisiae*. The figure demonstrates the production of IP8 from IP3. IP3 = inositol-1,4,5-trisphosphat; IP4 = inositol-tetrakisphosphat; IP5 = inositol-pentakisphosphat; IP6 = inositol-hexakisphosphate; IP7 = diphosphoinositol-(1,2,3,5,6)-pentakisphosphate; IP8 = bis-diphosphoinositol tetrakisphosphate; PP = diphosphate. The information in this figure is based on Ref. [57] and [73].

Further, it has been shown that the levels of ATP, ADP, and PP-IPs are halved compared to P_i-repleted conditions. Both enzymes Kcs1p and Vip1p for IP7 and IP8 production, respectively with a K_m value of ~ 1.5 to 2 mM are susceptive to a decrease of P_i below 1 to 2 mM in the medium [82].

SPX domains are inositol pyrophosphate (InsP) receptors providing an InsP binding surface formed by a P_i binding cluster and a lysine surface cluster [67], [90]. This domain was first identified in yeast and is ~ 200 amino acids (aa) long. The structure of this hydrophilic domain is variable, but it has three well-conserved regions of 30-40 aa. The domain is often located at the N-terminus. Moreover, the SPX domain interacts with various proteins and a key role in P_i homeostasis could be shown in yeast [91]. When InsP is binding to such a SPX domain the activity of SPX-associated protein is modulated by changing the protein structure [92]. Depending on the target protein this interaction can have an activating or repressing effect [89]. Furthermore, the interaction of the InsP and SPX domain is found in many eukaryotes such as fungi, plants, and humans [92] and mutations in the encoding gene generate various phenotypes [71], [85], [93]. A lot of proteins involved in P_i homeostasis and polyP production are regulated by a SPX domain. Another molecule that directly interacts with this domain is Spl2p (Squamosa promoter-binding-

like protein 2). It is expressed under low P_i conditions and can inhibit for example the activity of the low-affinity transporters Pho90p and Pho87p [71].

1.5.2 Polyphosphate formation by VTC complex

The eukaryotic vacuolar transporter chaperon (VTC) complex is the polymerase synthesizing polyP by using P_i and translocating polyP across the vacuole membrane. The VTC complex consists of five subunits (Vtc 1-5) and is presumably dependent on a proton gradient created by the V-ATPase [94], [95]. Additionally, to the polyP synthesis, the VTC complex has been demonstrated or is suspected to be involved in other cell activities *e.g.* in the stabilization of the vacuolar V-ATPase or vacuole fusion, and autophagy [95], [96]. The VTC complex is localized in the vacuole membrane but was also found in a peripheral location presumed to be the endoplasmic reticulum (ER) [97]. In eukaryotes, polyP is mainly accumulated as granules in the vacuoles [90]. Although the process of polyP production is described in a broad range of eukaryotic organisms, the VTC complex is the only isolated eukaryotic-type polyP-synthesizing enzyme so far [98]. In yeast, this complex has two sub-complexes consisting of at least three subunits: Vtc1p, Vtc2p, and Vtc4p or Vtc1p, Vtc3p, and Vtc4p. The first subunit combination (Vtc1p, Vtc2p, Vtc4p) tends to be located in the ER and/or in the cell periphery while the second one (Vtc1p, Vtc3p, Vtc4p) is more enriched in the vacuole. Under P_i starvation conditions both isoforms are localized to the vacuole [99]. The subunit Vtc1p has a trans-membrane domain whereas Vtc2p, Vtc3p, and Vtc4p harbor additional cytoplasmic SPX and triphosphate tunnel metalloenzyme domains [90], [96], [97]. It was predicted that the transmembrane domains have at least nine transmembrane helices [90]. The tunnel domain of Vtc4p is mainly responsible for synthesizing polyP. The synthesis is driven by the transfer of P_i from ATP to the new polyP chain [90], [98]. The catalytic activity requires Mn^{2+} located in the active site [98]. Vtc2p and Vtc3p are assumed to be subunits with a regulatory function but without any catalytic activity. Apart from that they are highly homologous to Vtc4p [94]. Vtc1p is a small protein that resembles the transmembrane domains of the VTC complex and contains no cytoplasmic domain that is homologous to those of the other subunits [94], [97]. All transmembrane domains combined form a channel transporting the growing polyP chain through the vacuole membrane into the lumen [90]. Further, it could be shown that the subunits Vtc1p, Vtc3p, and Vtc4p assemble in a stoichiometric ratio of 3:1:1 and form a pentamer. The cytosolic SPX domains regulate the polyP synthesis by binding inositol pyrophosphate [90]. Despite the higher potency of IP8, 5-IP7 is the primary activator of the VTC complex binding to the SPX domains at around 500 nM [84]. Recent studies have shown that Vtc1p and Vtc4p are essential for the generation of polyP whereby a

deletion of Vtc2p or Vtc3p only leads to a small reduction of polyP synthesis [90]. All these subunits are strongly induced upon P_i starvation. In yeast another sub-complex called Vtc5p was found but only in the vacuolar membrane. It interacts with the VTC complex and accelerates polyP synthesis, but it is not essential for it. This subunit also has a SPX domain. Furthermore, it differs from the other subunits as it is not strongly activated during P_i starvation [96], [97].

1.5.3 Polyphosphate degradation

S. cerevisiae has eight known phosphatases that are involved in P_i homeostasis and polyP degradation. Five of them (Ppn1p, Ppn2p, Ppx1p, Ddp1p, Pho8p) are intracellularly active [100], [101]. Since the highest amount of polyP is located in the vacuole the most important polyphosphatases are the endopolyphosphatases Ppn1p and Ppn2p, which are also located in the vacuole and mobilize P_i by cleaving polyP [102], [103]. Although these two enzymes are in the vacuole, are named similarly, and have equal functions, their sequences are unrelated [104]. Ppn2p cleaves polyP chains up to lengths of 60 P-subunits whereas Ppn1p fragments high molecular polyP into low-molecular ones, especially in the presence of Co^{2+} and Mg^{2+} [105]. Additionally, Ppn1p shows an exopolyphosphatase activity cleaving orthophosphate from the ends of polyP chains [105]. Accordingly, it is important for determining polyP chain length in the stationary phase [104]. Its exopolyphosphatase activity is predominant in the presence of Co^{2+}, while its endopolyphosphatase activity is predominant in the presence of Mg^{2+} [106]. Further, Ppn1p was also found in the nucleus, and mitochondrial membrane [103]. Ppn2p belongs to the phosphoprotein phosphatases (PPP) and is activated by Zn^{2+} ions [104]. The non-specific P_i-repressible alkaline phosphatase Pho8p is also located in the vacuole [101], however, it rather dephosphorylates phosphohistones and phosphopeptides [107]. As soon as P_i is released by the mentioned enzymes, the low-affinity P_i exporter Pho91p transports the orthophosphate from the vacuolar lumen to the cytosol which makes it accessible to other intracellular processes [68], [69]. It is a Na^+/P_i symporter and is localized in the vacuole membrane [93], [108]. In contrast to a lot of other genes involved in P_i homeostasis, this transporter is independent of P_i concentration and Pho4p activity [87] but is regulated by its SPX domain [76], [93].

Ppx1p, another exopolyphosphatase in yeast is located in the cytosol and the soluble fraction of mitochondria. It hydrolyzes shorter polyP chains including polyP3 more effectively [103] as its K_m value decreases with increasing polyP chain length [109]. The activity of Ppx1p is dependent on divalent cations like Mn^{2+}, Mg^{2+} and Zn^{2+}. However, the function of this enzyme is not entirely clear since polyP accumulation in the cytosol of yeast has not been described [49]. Additional to Ppx1p, the diphosphoinositol polyphosphate phosphohydrolase (Ddp1p) an endopolyphosphatase,

is also located in the cytosol [110]. Although Ddp1p mainly hydrolyzes InsPs and nucleotide analogs, such as diadenosine hexaphosphate, it possesses a robust endopolyphosphatase activity as well. It is fluoride-sensitive and becomes inactive in the presence of Mn^{2+} [111]. Finally, three more polyphosphatases can be secreted. The acidic polyphosphatases Pho5p and its analogs Pho11p and Pho12p are normally located in the periplasm between the plasma membrane and the cell wall degrading diverse organic P_i-containing compounds extracellularly [83], [112].

1.6 Scope and outline of this thesis

In this thesis, a contribution to solving the broken P_i cycle problem by using yeast for P_i recycling is presented. Recycling and reuse of various waste streams were investigated regarding their applicability as alternative P_i sources for the production of polyP to prevent its discharge into water bodies. Since the options for increasing the polyP chain length and the polyP yield from yeast using process optimization are limited, possibilities for improving production using metabolic engineering were investigated. By combining P_i-rich waste streams as a source of P_i and using the improved strains, the process for polyP production using microorganisms can be made competitive and commercially viable in the future.

In chapter 1 the reader is introduced to the natural P_i cycle and the problems which arise when humans interfere with it (chapters 1.1 to 1.2). Further, polyP, its industrial applications (chapter 1.3), and the polyP metabolisms in *S. cerevisiae* (chapter 1.5) are described. The section on polyP metabolism considers both the genes that are involved in P_i homeostasis and the genes involved in polyP degradation. Chapter 2 describes all experimental approaches that were performed to generate the results described in chapters 3 to 1.

In chapter 3, plant-derived phytate is used as an alternative P_i source for the production of polyP by *S. cerevisiae*. Nine different plant extracts containing mobilized P_i are used as P_i sources to produce polyP. Further, polyP-rich yeast extract is produced and tested as a food additive in sausages during an application test.

In chapter 4, an industrially derived waste stream was tested for P_i recycling as an alternative source. Therefore, P_i-rich wash waters, waste streams generated during chemical polyP synthesis in industry, are tested as a P_i source. The produced polyP is characterized and a method to adjust the counterion is presented.

After testing different P_i sources and analyzing the polyP content, in chapter 5, producing *S. cerevisiae* strains are optimized via metabolic engineering to improve the productivity including polyP content (% (w/w) polyP/cdw), yield (polyP/P), and titer (g polyP/L feed medium) and the average chain length of polyP. PolyP-degrading enzymes and genes with a predicted negative effect on polyP production are deleted. Afterward, the generated strains, the production process, and the generated polyP are characterized.

The general discussion in chapter 6 summarizes the findings of this doctoral thesis and places them in a larger context. The chapter deals first with the numbers and costs of the global and German P_i market and subsequently with the prerequisites and challenges for establishing a circular P_i economy. These range from specific problems of individual P_i recycling strategies to challenges that politics and society as a whole must face.